Entomology in Focus 5

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Triatominae – The Biology of Chagas Disease Vectors

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Foreword

As the reader probably knows, triatomine bugs pose a menace to the large and diverse rural communities inhabiting poor regions of Latin America, due to the fact that they can house *Trypanosoma cruzi,* a parasite that acts as the etiological agent of Chagas disease. The transmission of this parasite to humans by triatomine bugs happens inside their domestic premises where bugs find shelter and the blood of their hosts. It is undeniable that the dramatic epidemiological consequences of this reality rely upon the region's social inequalities, which stem from and are reinforced through its poor development strategy, based mostly on precarious extractivist economies, the absence of effective public policies, and a growing environmental degradation due to predatory exploitation of commodities. Nevertheless, as triatomine bugs are present in sylvatic habitats throughout the region, their endemicity, as well as that of the different strains of the parasite, represents a source of a permanent public health concern that requires rational and sustainable control methods to be developed. Controlling Chagas disease relies fundamentally on vector control, and vector control, in turn, relies on scientific knowledge. This book represents our attempt to consolidate the current knowledge on triatomine bug biology, covering the diverse topics that have called the attention of scientists to date.

Among the first insect models to be studied from a physiological point of view by Wigglesworth, triatomines are amenable to diverse methods of experimental manipulation. This fact can be confirmed through the pages of this book, where experimental evidence on their developmental, cellular, physiological, genetic, and ecological characteristics have been reviewed by most authoritative scientists studying them. We have attempted to cover every main research field on which we perceive that scientific progress has been reached on triatomine bug biology. Therefore, we would like to express our deep gratitude to every contributor that has helped us build such a broad and profound collection of knowledge on the biology of these vector insects, as well as to the reviewers that have significantly improved all chapters with their fundamental inputs. We would also like to acknowledge that it is frequently impossible to cover all relevant scientific findings on such a broad scope; we apologize for any involuntary exclusion in this first edition of the book. We look forward to improving it if an opportunity for updates appears in the future.

We expect this book to help scientists, as well as students working on these vectors, to have access to a consolidated revision on the biology of these relevant insects. As such, we consider that it will serve as a textbook for medical entomology courses: a first on the topic. We express our joy for having reached such an exciting goal and hope it will represent a significant contribution to the field, and, consequently, to a more holistic and rational approach to bug control. We would like to dedicate this book to our supervisors, who guided the start of our careers and gave us most of the tools we use in our scientific paths. And, fundamentally, to the legions of victims of Chagas disease, the greater part of whom remain deprived of their most basic rights: they alone constitute millions of reasons behind this book.

Preface

Modern systems biology has much to owe to the concept of "model species." This label refers to organisms that are privileged for the study of particular biological phenomena because they offer powerful tools that allow answering questions at multiple levels of analysis. Decades of research on these animals have led to the establishment of standardized procedures for raising them in the laboratory under controlled and accessible conditions, as well as to the conception of behavioral protocols that allow addressing biological phenomena in a tractable way. Typical examples are the fruit fly *Drosophila melanogaster* and the zebra fish *Danio rerio*, which ally the possibility of coupling behavioral studies with invasive cellular methods and the possibility of using transgenesis to explore functional principles of their biology.

Yet, the concept of "model species" brings a potential risk for modern biology, namely, the dominance of a handful of species, which are not representative of the rich spectrum of biological solutions that evolution has implemented in animals. As an example, the actual list of model organisms for biomedical research of the US National Institutes of Health (NIH) cites only eight animal species, among which fruit flies are the only insects considered [1]. This situation may induce research policies with narrow perspectives and with little space for diversity. It is difficult to see how such a trend could promote our understanding of rich biological solutions implemented by evolution to cope with variable environments.

This is particularly evident in the case of insects, which are the largest and most diverse group of organisms on Earth. There are approximately 30 orders with the number of species estimated reaching nearly 5.5 million, of which only 1 million have been named [2]. These animals are remarkably successful in evolutionary terms, as they have colonized practically all existing habitats on earth, including the extreme ones. Clearly, a single species cannot claim hegemony in terms of its value for improving our understanding of this success. On the contrary, enlarging the spectrum of species studied through a comparative approach is mandatory. From this perspective, a book on the biology of Triatominae is a fundamental contribution. Considering the specificities of the lifestyle of these insects – based on their blood feeding habits and their association with flagellates that are responsible for

one of the most severe world diseases, the Chagas disease – a book summarizing the state-of-the-art research in these animals, for which ca. 150 species are known, is highly welcome. It enriches our view on the basic principles of insect biology and represents a unique opportunity to appreciate the richness of biological solutions implemented by triatomine species. The editors of this book should thus be congratulated for putting together a chapter collection that covers a broad spectrum of areas, from behavior and physiology to biological control and genomics.

This collection has, in addition, a strategic importance that will make it indispensable for establishing sanitary policies preventing the spread of Chagas disease in the Americas. This disease, which affects millions of people, is caused by the flagellate *Trypanosoma cruzi*, which is hosted by the insects' digestive system and which penetrates into the circulatory system when the bugs bite their hosts, ingest blood, or defecate on them. Typically associated with poverty and social marginality, the disease poses problems that require an integral understanding of the biology of the vector as an essential step toward the implementation of successful control strategies. Clearly, such strategies require a multidisciplinary approach, and the book succeeds in providing a multi-facetted perspective for addressing this endeavor. The convergence of contributions on biological control and insecticide resistance with chapters on Triatominae behavior, sensory physiology, and neurotransmission, to cite some examples, paves the way toward integral and intelligent management of Chagas disease.

The book does justice to the historical importance of kissing bugs for biological research. Several decades ago, Vincent B. Wigglesworth (1899–1994), who was a pioneering figure in the field of insect physiology and insect endocrinology, established *Rhodnius prolixus* as a key organism for insect physiology. His work on this insect led, among others, to the discovery of juvenile hormone [3], the hormone that prevents metamorphosis in insects, and elucidated some of its roles, such as its role of guidance for the molecular action of ecdysone by preventing switching-on of metamorphic-specific genes. Most of his findings are summarized in his book *The Principles of Insect Physiology* [4], which is a classic among scholars interested in entomological research, and which can now be updated by the 20 chapters of the present book.

The book appears in a particular moment of the history of biological research on Triatominae. In consonance with the *Zeitgeist*, the genome of *R. prolixus* has been recently sequenced and made available [5]. Its publication revealed immune signaling pathways that display major departures from the canonical network, and large gene expansions related to chemoreception, feeding, and digestion that have facilitated the adaptation of triatomines to a blood-feeding style.

From the perspective of a researcher working on honeybee perception and cognition, the publication of the genome of the honeybee [6] was a significant event that promoted new research questions and induced the development of novel molecular tools to address numerous problems in honeybee biology. Moved by this fast progress, 5 years after this publication we felt compelled to edit a book providing an integrative review on the behavior and physiology of honeybees [7]. Coincidently, 5 years after the publication of the genome of *R. prolixus*, a new book on the biology

of Triatominae sees light, moved by a similar exponential growth in knowledge. Not surprisingly, therefore, molecular approaches are present in many chapters, from sensory physiology to evolution and the development of "omics".

On a personal note, a honeybee researcher writing the preface of a seminal book on kissing bugs may be an intriguing fact for its readership. Yet, despite my historical commitment to social insect research, I always had a very close relationship with triatomines, as shown, for instance, by the two works on sensory physiology of *Triatoma infestans* in which I had the chance to participate [8,9]. I did my PhD studies under the supervision of Josué Núñez, who returned to Argentina after the military dictatorship (1976–1983) with the goal of establishing a research line on the behavioral physiology of the main triatomine vectors. At that time, Núñez offered me the possibility of starting a PhD on *T. infestans*, which I declined, asking him, in return, to work on aspects related to the sensory physiology of another species for which he had made fundamental contributions, the domestic honeybee *Apis mellifera*. Núñez accepted my request, and I had the chance to share the laboratory with several of his notable students working on the behavior of kissing bugs, many of which became important leaders in their field of study. Although I never regretted this election, this preface may serve as a personal tribute to kissing bugs, to their fundamental and historical value for biological research, their socioeconomic and medical importance, and an acknowledgment to the necessity of watching beyond own research frontiers.

The book is a magnificent update to understand the essential aspects of the biology of kissing bugs and serves, in addition, to delineate questions that need to be tackled in future research. It should, therefore, call the attention of a broad spectrum of scholars in diverse disciplines, well beyond those interested in Chagas disease vectors. As usually in science, inspiration may come not from the narrow area in which one tends to be confined, but from farther, unsuspected horizons. I am convinced of the inspirational potential of this book, and I am therefore thankful to the editors for the unique privilege of writing this preface.

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Introduction

Triatomines are the vectors of *Trypanosoma cruzi,* the etiological agent of Chagas disease, one of the most important infectious diseases in the Americas, affecting about 6–7 million people (WHO 2015). Infection by *T. cruzi* has been reported to occur from the southern United States to southern Argentina (between latitudes 40°N to 45°S). Approximately 30% of infected people develop the disease, while the remainder of individuals present the indeterminate form. Among symptomatic individuals, the cardiac form of Chagas disease is the main clinical manifestation, but digestive manifestations are also important, with impairment of esophageal and colonic peristalsis. According to an assessment in the 1990s by the World Bank, the calculation of "Disability-Adjusted Life-Years" (DALYs), with respect to other communicable diseases in the Americas, showed that only diarrheal diseases, respiratory infections, and AIDS exceeded the economic losses resulting from Chagas disease (World Bank 1993; https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(18)32279-7/fulltext). Although it has high morbidity, and clinical manifestations require medical care, there is a good prognosis for the treatment of recent acute and chronic cases, with excellent results in terms of improved quality of life for chronic patients (Dias et al. 2016). In Brazil, only acute infections used to be notifiable, but notification of chronic forms has recently become mandatory. Currently, there is a recommendation to treat individuals who are asymptomatic or with mild symptoms in order to prevent the subsequent development of severe forms of the disease (www.in.gov.br/web/dou/-/portaria-n-1.061-de-18-de-maio-de-2020-259143078; Perez-Molina and Molina 2018). This practice enables a better assessment of the current importance of the disease through case notification, and requires the health system to accept and treat these patients, either by offering a treatment specific to *T. cruzi* infection or treating the symptoms associated with Chagas disease. This task is not at all easy: the disease has been historically neglected (www.who.int/neglected_diseases/diseases/summary/en/) as exhibited by the lack of clearly defined priorities for control strategies (Silveira and Matins 2014) and the failure to sufficiently educate medical health practitioners about the disease (Coura 1980).

Historically, the main route of *T. cruzi* transmission to humans is through triatomines. However, as the parasites circulate in the blood, transmission can also occur through blood transfusion, congenitally, breast-feeding, organ transplantation, laboratory accidents, and handling infected animals, as well as orally through ingestion of contaminated food (Carvalho et al. 2015). The typical profile of the people most affected by Chagas disease includes low socioeconomic status and residence in rural areas. Typically, such people live in countries whose economic systems encourage and maintain social inequality. Their relatively simple houses, built with natural materials (such as clay, wood, straw, etc.), are clearly at risk of infestation, and allow colonization, by triatomines (Dias and Coura 1997). Recently, with the improvement and success of vector control programs, and a subsequent decline in intradomestic vector-borne transmission, ingestion of contaminated food has become the main form of transmission (Dias et al. 2016). With the increased movement of people between countries related to globalization, Chagas disease, which was originally only found in the Americas, is a problem today in the non-endemic countries of Europe, Asia, and Australia (Klein et al. 2012), as well as non-endemic regions of the United States (Bern et al. 2020).

Triatomines are widely distributed throughout the Americas, occurring in many and diverse environments, which is an indication of the different adaptive processes these insects have undergone to different conditions. The insects are also associated with numerous vertebrate species, which are their sources of food. They are hematophagous in all their developmental stages (nymphs and adults) (Lent and Wygodzinsky 1979). Hematophagy requires physical proximity to the vertebrate host species that are their blood meal sources, and the physiological ability to process different types of blood (Pereira et al. 2006). In sylvatic environments, the distribution of triatomines is focal and dependent on the biology of the vertebrate hosts with which they are associated. In unstable ecotopes, where environmental conditions are variable and the presence of vertebrate hosts is sporadic, triatomine colonies are comprised of only a few individuals. In stable ecotopes, with little fluctuation of environmental conditions, and the frequent occurrence of vertebrate food sources, triatomine colonies can grow to very high densities (Diotaiuti 2009). The latter is typically what occurs when triatomines colonize and infest artificial environments associated with humans. Triatomine species physiologically capable of adapting to these new conditions can form even larger colonies than those occurring in sylvatic environments, due to an increase in their reproductive capacity related to increased food availability. The ability to colonize modified environments created by humans, and obtain blood meals from domestic animals and humans, characterizes the triatomine species as having the greatest epidemiological importance for Chagas disease (Pereira et al. 2006).

Infection with *T. cruzi* is enzootic in wild animals, especially affecting a wide range of small mammals. In sylvatic environments, oral infection either through ingestion of an infected triatomine or another infected mammal is probably the main form of transmission (Jansen et al. 2018). Infectious forms of the parasite, however, are also eliminated in the feces of triatomines, which was once the main form of human transmission of Chagas disease. Infectious forms of the parasite are

eliminated during or immediately after a triatomine takes a blood meal, and subsequently penetrates the skin either through a wound or the mucous membranes of the eyes or mouth. Several factors are involved in this complex process that culminates in the transmission of *T. cruzi*, for example, the preference of triatomines for feeding on human blood, how quickly triatomines obtain their blood meal without being noticed and return to their hiding place, and how rapidly the infected feces of the triatomine are eliminated onto their vertebrate host (Martins 1968). Many factors need to coincide for parasite transmission to take place, which explains, for example, why not all residents of the same house become infected, despite living in the same conditions for many years. These features define the classic route of human-vector transmission of Chagas disease, associated with high-density triatomine infestations of households, in which the insect colonies often have more than 1000 individuals! That disadvantaged people serve as a source of blood meals throughout their lives both literally and metaphorically epitomizes the social inequalities that exist in our societies.

In Brazil, the endemic area of Chagas disease overlaps with the area of triatomine occurrence, especially the triatomine species capable of colonizing artificial human-made environments (Silveira et al. 1984). This region corresponds to the ecological "dispersion corridors" of South America, the "diagonal" of open savanna-like ecoregions – the "corridor" – that includes different biomes of great epidemiological importance: the Chaco, Pantanal, Cerrado, and Caatinga (Forattini 1980). There are many different species of triatomines each associated with a wide range of different ecotopes, such as palm trees, tree trunks or their hollows, bromeliads, rocks, bird nests, or the burrows of small mammals. Triatomines feed on diverse sources of blood, including both warm- and cold-blooded vertebrates, and even the hemolymph of other arthropods (Diotaiuti et al. 1993; Lorosa et al. 2000). This has resulted in triatomines adapting in different ways to the environment, often leading to the formation of distinct populations or even new species. During these adaptive processes, triatomines undergo physiological, anatomical, genetic, and behavioral changes, investigation of which may enable us to discover new ways to control the transmission of *T. cruzi* to humans.

Based on knowledge of some organisms such as vertebrates, insects, and other animals, Amorim and Pires (1996) proposed the existence of 47 main centers of Brazilian Neotropical endemism, resulting from the fragmentation of spaces corresponding to the areas of the Amazon and the Atlantic Forest. These very large environments have particular features, which may shed light on the diversity of triatomines, demanding the use of particular tools to understand the processes involved. An interesting example could be the study of the genus *Rhodnius*, which is associated with different species of palm trees and which probably originated from the Orinoco region (Abad Franch et al. 2015). *Rhodnius prolixus* is the main vector of *T. cruzi* in the countries of northern South America. This triatomine species was also accidentally introduced into Central America, where it was also a very important vector until it was eliminated as part of an intergovernmental control initiative (Hashimoto and Schofield 2012). Among the well-known 20 species of *Rhodnius* (Justi and Galvão 2017), there is a species complex (Barrett 1988) that

includes four taxa, which are of particular interest to researchers: *Rhodnius prolixus, R. robustus, R. neglectus*, and *R. nasutus*. According to Monteiro et al. (2003), the occurrence of *R. prolixus* is restricted to northern South America, while *R. robustus* occurs in a wider area and has a greater genetic diversity, possibly related to the centers of endemism determined by Morrone (2006). The other members of the species complex inhabit the diagonal of open savanna-like ecoregions described by Forattini (1980): *R. nasutus* is associated with the Caatinga, and *R. neglectus* with the Cerrado. The influence of the microenvironment is clearly demonstrated in the association between *Rhodnius* species and palm tree species. In the semi-arid region of Ceará, *Rhodnius nasutus* colonizes two species of palm trees, namely, babassu (*Attalea speciosa*) and carnauba (*Copernicia prunifera*). Babassu occurs in mountainous regions, with similarities to the Atlantic Forest (Cavalcanti 2005). These latter trees are leafy palms that provide a stable microclimate throughout the year, compared to the external environment. Under these conditions, *R. nasutus* have a dark brown color, and morphometric studies have shown that they are larger than conspecifics captured in carnauba palm trees. Carnauba trees grow in the arid plains of the Caatinga. The leaves of these palm trees are inserted into the trunk, allowing humidity and temperature to fluctuate according to variations in the external environment. *R. nasutus* that colonize carnauba palm trees are smaller than those found in babassu, with a reddish color similar to the bracts of the leaves of the palm trees that they inhabit. Characterization with microsatellites shows that the *R. nasutus* found in babassu have less genetic variability than those captured in carnauba. In this example, there were several intra-specific changes, depending on the environment (Dias et al. 2008). For instance, greater availability of blood meal sources in babassu, allows triatomines to have a larger size and greater reproductive capacity, and, consequently, larger colonies. In addition, by adapting their color to the colors of the substrate where they live, triatomines have increased protection. The greater genetic variability of the *R. nasutus* that live in carnauba is probably due to their greater dispersal ability, enabling larger effective population sizes, as these latter insects are very active and frequently fly between palm trees. It is not known whether this migration is stimulated by the limited blood meal availability associated with carnauba. Specimens of *R. nasutus* found in households are reddish, which indicates that they come from the carnaubas.

Another interesting example in Brazil of the adaptive capacity of triatomines to the environment is the *brasiliensis* species complex, which is associated with the semi-arid region and is comprised of eight taxa: two subspecies*, Triatoma brasiliensis brasiliensis* and *Triatoma brasiliensis macromelasoma* (Costa et al. 2013)*,* and six species*, Triatoma lenti, Triatoma juazeirensis, Triatoma melanica, Triatoma bahiensis* (Mendonça et al. 2016)*, Triatoma sherlocki* (Mendonça et al. 2009), and *Triatoma petrocchiae* (Schofield and Galvão 2009; Oliveira et al. 2017). *Triatoma brasiliensis brasiliensis* is the main native *T. cruzi* vector in northeastern Brazil, usually being found in the deep cracks formed between large granite rocks, where they reproduce and obtain their blood meals, mostly from rodents (Alencar 1987; Bezerra et al. 2018). On occasion, in the absence of rocks, this subspecies can be found in the cactus *Pilosocereus gounellei* (Valença-Barbosa et al. 2014). This

subspecies is very agile, fast, opportunistic; it attacks animals and unwary people in sylvatic environments. The Brazilian semi-arid region has a dry and hot climate, and the rocks where *T. b. brasiliensis* lives can reach very high temperatures during the day (Catalá et al. 2015). At dusk, when the temperature is cooler, the insects leave their hiding places, and stay on the surface of the rocks, and return to their hiding places after 9 pm. *Triatoma brasiliensis*, therefore, modulates its behavior in the field, as reproduced by Guarneri et al. (2003) under laboratory conditions. However, on cooler and cloudy days, hungry nymphs of this species attacked us in the middle of the morning, an unusual behavior among triatomines, which usually blood-feed in the early evening (Catalá et al. 2015). The artificial environment is infested by sylvatic colonies, which easily adapt to intradomestic conditions, which mimic the microclimatic characteristics of their natural environment (Lorenzo et al. 2000). Experimentally, *T. brasiliensis* adults are not considered to be "good flyers" (Soares 1997). This is confirmed by observing them in the field, during the period when they leave their cracks and remain on the rock surface. Theoretically, only 1% of individuals are expected to disperse by flight; however, under field conditions, even this small proportion still represents a large number of insects, some of which will invade and colonize human-associated environments. A study carried out in the state of Ceará used microsatellites to compare insects collected in the sylvatic and household environments and in households (intradomicile and peridomicile) on five separate occasions between 2009 and 2015. After spraying with residual insecticides, the insects showed no cluster formation, that is, an intense exchange of insects among different environments (Belisario 2018). Practically, this finding has great significance for vector control, as it demonstrates constant reinfestation of households by sylvatic triatomines after insecticide use. The continual re-application of insecticide requires a great deal of effort and high levels of vigilance (i.e., entomological surveillance) in order to prevent reestablishment and growth of colonies inside households. Re-infestation, or even the persistence of domestic colonies, after insecticide spraying of domestic environments is the biggest challenge faced by triatomine control.

The "domestic unit" is considered as the focal epidemiological unit for control activities against triatomines, since there is a high level of triatomine migration between the surroundings of a house and its interior (Brasil/SUCAM 1980). That is, spraying involves the entire domestic unit, regardless of whether infestation is intra- or peridomestic (Souza 2019). The peridomestic environment consists of chicken coops, pig sties, storerooms, piles of firewood, stones or bricks, fences, or any structure where triatomines can find shelter and food. Because of the complexity of these habitats, triatomines are not easily found within them (Cecere et al. 2004; Diotaiuti 2009; Espinoza-Echeverria 2017). The development of devices to facilitate the capture of triatomines, especially in the peridomestic environment, has long been desired (Mota et al. 2014), but requires further research. In addition, insecticide spraying does not ensure that all triatomines will have contact with the insecticide, and, if they do, that the exposure is sufficient to cause death, that is, there are insects that survive and reestablish the colony (Gurtler et al. 1994; Gurtler 2009). For

T. brasiliensis, this reestablishment takes place within an average period of 12 months (Diotaiuti et al. 2000).

Among the 151 species of triatomines previously described (Justi and Galvão 2017), less than 20 are capable of colonizing the artificial environment. For these latter species, chemical control is able to drastically reduce indoor infestations, especially the density of insects inside households (Silveira 2011). However, such control is not as successful in the peridomestic environment, and the transmission cycle persists in the immediate vicinity (Bezerra et al. 2020). Entomological surveillance, therefore, is essential to control peridomestic colonies of triatomines, and prevent colonization of households.

Triatoma infestans was the main vector in Brazil, Argentina, Bolivia, Chile, Paraguay, and Uruguay (i.e., the countries that form the "Southern Cone" of South America) (Silveira 2002), and it is easy to maintain their colonies in laboratory insectaries. Originating mainly in the Bolivian inter-Andean valleys (Forattini 1980), this species was passively dispersed over wide areas, without, however, adapting to novel local sylvatic environments. Pereira et al. (2006) demonstrated that the species has a better blood-feeding efficiency, finishing their blood meal more quickly than other triatomine species. This ability apparently explains, for example, the competitive success of *T. infestans* and its expansion into areas infested by indigenous triatomines, such as *Panstrongylus megistus* or *T. brasiliensis*. However, due its limited occurrence in sylvatic environments, it can be eliminated through spraying with residual insecticides, which was achieved through the "Initiative for Elimination of *T. infestans* in the Southern Cone Countries" (Silveira et al. 2002). Chile, Uruguay, and Brazil have already been certified for elimination of *T. cruzi* transmission by this triatomine species, and a great deal of success can be recognized in other countries in the Southern Cone region. Unfortunately, in Brazil, this certification has been confused with elimination of transmission to humans by *native* triatomine vectors, which has had a very negative impact on initiatives to control native triatomine species (Abad-Franch et al. 2013), despite the acknowledged importance of *P. megistu*s and *T. brasiliensis* in areas where *T. infestans* has never been present. Only in recent years has there been any effort (but clearly insufficient) to maintain entomological surveillance in the areas recognized as endemic for *T. cruzi* transmission by native triatomine vectors.

The importance of flight dispersal as a mechanism of household colonization was recognized early (Lehane and Schofield 1976). In the last few decades, the invasion of households without colonization by adult triatomines has also been associated with episodes of *T. cruzi* transmission (Aguilar et al. 2007). In fact, this form of transmission has possibly always existed, but it has perhaps just become more evident now, given the current lower levels of infestation of households in endemic areas. Alternatively, it is possibly due to environmental changes, such as deforestation or climate change.

Similarly, oral infection is now statistically the major route of transmission. Food contamination resulting from contact with infected triatomines, accounted for 74.5% of acute cases notified in Brazil in the period between 2007 and 2019 (SVS/MS 2020). With the discontinuation of control programs, and the resulting limited

capacity of health professionals to carry out diagnosis of the disease, the epidemiological information collected has decreased in quality in recent years. Therefore, nowadays, there are no longer accurate and up-to-date data on the number of acute cases (Abad-Franch et al. 2013).

The very bad news was the finding of *T. infestans*' resistance to insecticide pyrethroids (Vassena et al. 2000), subsequently proven for different categories of insecticides (Pessoa et al. 2015). Considered unlikely, given the fact that triatomines have a long evolutionary cycle (one annual cycle, but rarely two), and considering the good results found with insecticides, the previous information on the occurrence of resistant *R. prolixus* in Venezuela (Rocha e Silva 1979) was neglected. However, in the Bolivian and Argentinian Chaco, a region where insecticide resistance rates have reached the highest levels (Gomez et al. 2016), *T. infestans* infestations persist. Studies have shown that *T. infestans*' resistance to pyrethroid insecticides is autosomal and an incompletely dominant character (Gomez et al. 2015). The physiological mechanisms involved differ and are polygenic (Pessoa et al. 2015), but there are many things still to be learnt about the genetic basis of insecticide resistance. In the field, the insecticide resistance of *T. infestans* has an irregular and patchy distribution, with levels that vary between different structures close to one another within the same peridomestic environment, and appears to have originated from the preexistence of resistance in sylvatic triatomines not previously exposed to insecticides (Espinoza-Echeverria et al. 2017). In Brazil, REMOT – the Network for Monitoring of Triatomine Resistance to Insecticides – was created to assess the susceptibility of native triatomine species (Pessoa et al. 2015). Insecticide resistance of *T. infestan*s in south Brazil has not been confirmed (Sonoda et al. 2009). In the state of Minas Gerais, data suggest a change in the susceptibility of *Triatoma sordida* that deserves to be investigated (Pessoa et al. 2014), while all populations of *T. brasiliensis* that were studied in the state of Ceará had a high susceptibility (Sonoda et al. 2010; REMOT 2020).

The study of triatomines has aroused the interest of scientists since the first description of the association of these insects with Chagas disease. One must consider the works of pioneering researchers, such as Arthur Neiva (Neiva 2010), Cesar Pinto (Pinto 1924), Eduardo Del Ponte (Del Ponte 1959), Emmanuel Dias (Dias 1956), Herman Lent and Pedro Wygodzinsky (Lent and Wygodzinsky 1979), and many others, on taxonomy, biology, and morphology, as well as contemporary studies, when the most modern techniques have been adopted. As an experimental model, knowledge of triatomines has been expanded, allowing reflection on the most diverse biological mechanisms, as can be seen in the scientific sophistication of the different chapters of this book. Entomological and epidemiological data, on the other hand, were the foundations for defining the importance, priority, and methodologies of control programs, which now incorporate environmental information, in an integrated understanding of the dynamics and complexity of the vectors of *T. cruzi*. Because of their epidemiological importance, and their greater ease of maintenance as colonies in insectaria, most research uses *T. infestans* and *R. prolixu*s as model organisms. In the last few decades, more studies have been undertaken on triatomine species previously considered to be of only secondary

epidemiological importance, but recognized with an appreciable role in the transmission to humans of *T. cruzi*. The continuing great challenge is to expand our understanding of the currently 151 recognized triatomine species, which can teach us a lot about life!

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Contents

Origin and Evolution of Triatominae

Christiane Weirauch

Abstract Triatominae, the kissing bugs, are one of the smaller subfamilies of the otherwise predatory hemipteran family Reduviidae, the assassin bugs. Substantial progress has been made during the past decades to resolve phylogenetic relationships between Triatominae and other reduviid subfamilies as well as relationships among the ~150 species currently classified as kissing bugs. Nevertheless, many open questions remain. While Triatominae are now shown to be part of an assassin bug clade that also comprises Stenopodainae and part of Reduviinae, it is still not conclusively established if Triatominae are monophyletic or paraphyletic, and there are uncertainties with regard to relationships between and within tribes of Triatominae. This chapter summarizes available information on the evolution of Triatominae, highlighting strengths and shortcomings of currently published phylogenetic hypotheses. It stresses the importance of densely sampled, data-rich, robust phylogenies to inform the classification of Triatominae and to severe as a framework for evolutionary investigations across the group.

Keywords Reduviidae · Triatominae · Kissing bugs · Evolution · Phylogenetic relationships

1 Background

With about 150 species in five tribes, Triatominae is one of the smaller of the mid-sized subfamilies of Reduviidae, the assassin bugs (Bargues et al. 2017; Georgieva et al. 2017; Justi and Galvão 2017; Schuh and Slater 1995). Reduviidae are the second largest family in the hemipteran suborder Heteroptera and currently comprise close to 7000 described species (Maldonado Capriles 1990; Putshkov and Putshkov 1985; Weirauch et al. 2014). They are found in all biogeographic regions, but their greatest diversity is in tropical areas around the globe. With the exception of Triatominae, Reduviidae are predators of insects and other arthropods and the group is known for

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their astounding range of both morphological and behavioral adaptations to capture prey including their ability to feed on chemically well-defended organisms such as millipedes and dangerous predators such as spiders (Forthman and Weirauch 2012; Soley and Taylor 2012). Specializations on particular groups of prey organisms are common in Reduviidae (e.g., ants, termites, or millipedes), but only Triatominae have evolved the ability to feed on vertebrate blood (Lent and Wygodzinsky 1979). Different lineages of Reduviidae have adapted to ecosystems and microhabitats ranging from mammal nests in the Sonoran Desert to decomposing logs in the Bornean rainforests and termite nests in the Isthmian-Atlantic moist forests.

Reduviidae are currently classified into 24 subfamilies, substantially more than any other heteropteran family, reflecting the morphological diversity in this clade (Forthman and Weirauch 2017; Weirauch et al. 2014). Unsurprisingly for a group of this size and complexity, the current classification of Reduviidae does not reflect evolutionary relationships within the group. Although the majority of subfamilies are well-supported as monophyletic groups, some are not (Hwang and Weirauch 2012; Weirauch and Munro 2009; Zhang et al. 2016a, b). The most dramatic example of a polyphyletic subfamily is Reduviinae—recognized exclusively by the absence of features present in the other subfamilies—that have been recovered in 11–13 different lineages in recent analyses (Hwang and Weirauch 2012). The past decade has seen a surge in published phylogenetic hypotheses for Reduviidae that have improved our understanding of the evolutionary history of the clade. However, phylogenies have included few exemplar species for most large clades and most analyses have not taken advantage of phylogenomic datasets and approaches, resulting in poorly supported hypotheses and substantial topological instability across different analyses.

This importance of densely sampled, robust phylogenetic hypotheses is also evident by the abundance of alternative hypotheses proposed for relationships of Triatominae to the predatory Reduviidae, the question if Triatominae are actually derived from a single common ancestor, and uncertainty on relationships among and within the five tribes of Triatominae (de Paula et al. 2005, 2007; Hwang and Weirauch 2012; Hypsa et al. 2002; Justi et al. 2016; Justi et al. 2014; Patterson and Gaunt 2010; Zhang et al. 2016a). Evidence-based answers to these questions are important, not only to streamline the systematics and classification of Triatominae, but also because they have bearing on our understanding of the evolutionary history of this group of important arthropod vectors, including the switch between predatory and hematophagous lifestyles, the age of hematophagy in this lineage, its ancestral geographic range and microhabitat, as well as the ancestral range of vertebrate host species.

2 Searching for the Closest Predatory Relative of Triatominae

The search for the closest predatory relative(s) of hematophagous Triatominae has been ongoing for several decades, complicated by the uncertainty if Triatominae are monophyletic, paraphyletic with respect to some predatory assassin bugs, or poten-

tially even polyphyletic, with different lineages within Triatominae (e.g., Triatomini, Rhodniini) related to different clades of predatory Reduviidae. Published molecular phylogenies support each of these three conflicting hypotheses, suggesting that the datasets and methods used to resolve this question have not converged on an optimal approach. The following section summarizes almost eight decades of studies that have attempted to reveal the closest predatory relatives of Triatominae and often at the same time have assessed if Triatominae form a clade or not. Much of the more current literature was recently summarized and illustrated in Monteiro et al. (2018) and reviewed by Otálora-Luna (2015) and is here only given as a brief overview.

Usinger (1943) did not question or test the monophyly of Triatominae, diagnosed Triatominae by the absence of dorsal abdominal glands, and in a pre-cladistic dendrogram based on morphology associated Triatominae with Reduviinae, Cetherinae, Salyavatinae, and Sphaeridopinae (Fig. 1a). Similarly, Lent and Wygodzinsky (1979) assumed that Triatominae were monophyletic, but pointed out that they share the absence of dorsal abdominal glands with some other Reduviidae, including Emesinae and Saicinae, and some genera currently classified as Reduviinae. They dismissed a potential close relationship between any of these taxa and Triatominae because of the lack of additional shared characters and putative synapomorphies. Lent and Wygodzinsky (1979) also pointed to the potential significance of the straight labium and the laterally inserted antennae found in Triatominae and certain other reduviids, in particular Epiroderinae (formerly Physoderinae). Epiroderinae are a circumtropical subfamily of small, brown assassin bugs usually found in decaying vegetation and rotting tree trunks where they feed on other insects (Weirauch et al. 2014). It has been suggested that at least one species of Epiroderinae may be facultatively hematophagous (Carcavallo and Tonn 1976), but there is no subsequent corroboration for this claim. Using explicit cladistic methodology and a large set of morphological data but coding taxa at the subfamily level and thus not testing their monophyly, Clayton (1990) recovered Triatominae as part of a polytomy that also comprises Stenopodainae, Peiratinae, Reduviinae, and three additional lineages with the remaining reduviid subfamilies except Hammacerinae. Into the 1990s, the monophyly of Triatominae had not been tested and there was no congruence on the hypotheses regarding the sister taxon of Triatominae.

Although initially not based on explicit phylogenetic methods and evidence, the notion that Triatominae may be polyphyletic (convergently similar morphology and/or behavior of unrelated taxa) and different lineages be most closely related to different predatory assassin bugs gained popularity in the late 1980s and even in fairly recent publications is sometimes still stated as a fact rather than a hypothesis (Bargues et al. 2017; Schofield 2000; Schofield and Dolling 1993; Schofield and Galvão 2009). Building phylogenetic hypotheses from 16S rDNA data, a fairly significant sample of Triatominae (Triatomini and Rhodniini only), and 15 predatory Reduviidae, de Paula et al. (2005) found support for the hypothesis that Triatomini are more closely related to Ectrichodiinae, Reduviidae, and Harpactorinae (part), while Rhodniini were recovered as sister taxon to Salyavatinae and Harpactorinae (part) (Fig. 1b). Using more substantial sets of molecular data, subsequent analyses

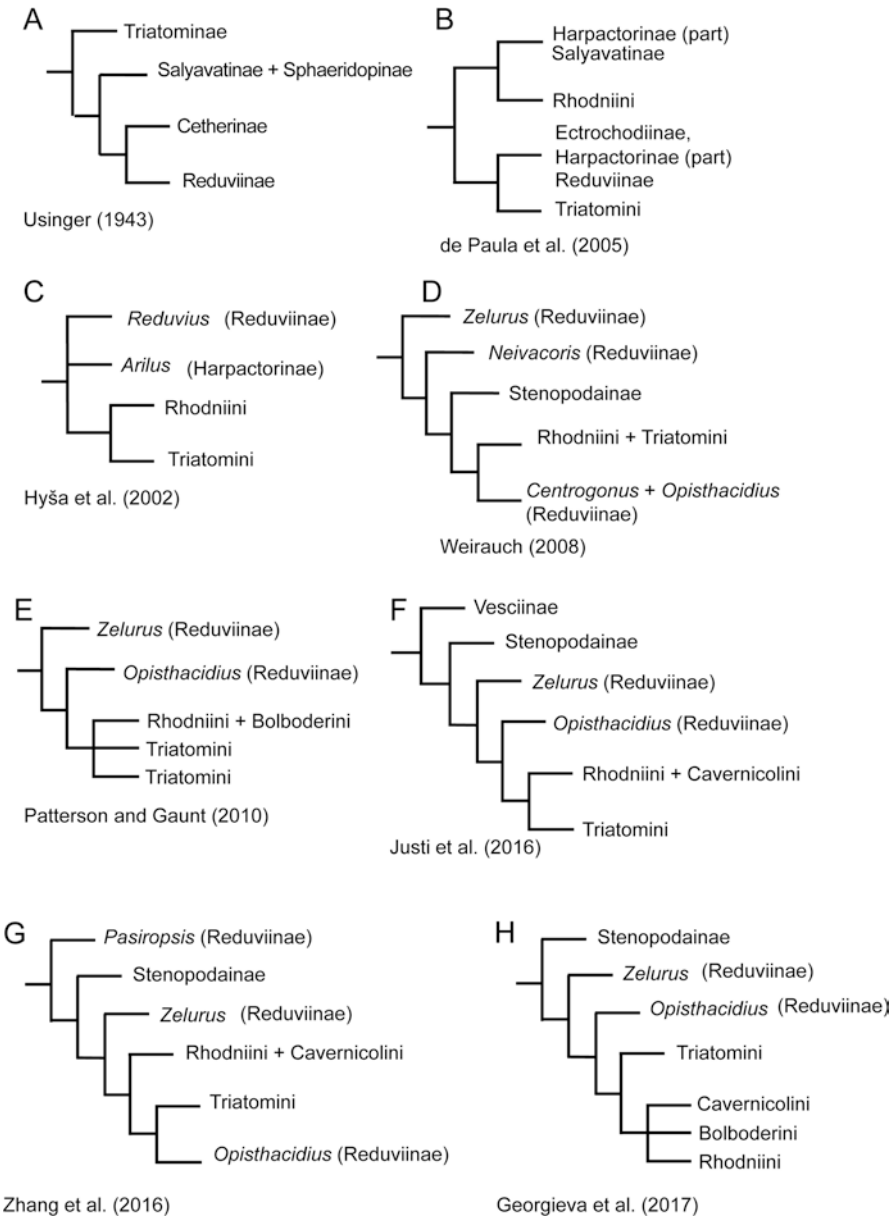

Fig. 1 Synopsis of phylogenetic hypotheses for Triatominae and closely related other Reduviidae

have never found similar results, suggesting that this topology may be an artifact based on insufficient data.

In contrast, the question if Triatominae are monophyletic or paraphyletic (lineage comprises most recent common ancestor but excludes some of the descendants) is

much less easy to settle. The latter would imply that the most recent ancestor of Triatominae also gave rise to certain presumably predatory taxa of Reduviidae that are currently classified in a different subfamily but would need to be included into the Triatominae to render the subfamily monophyletic. This scenario would infer a complete reversal from hematophagy to predatory lifestyle in the predatory assassin bugs nested within Triatominae, a somewhat unlikely hypothesis despite evidence that a number of kissing bug species engage in hemolymphagy under certain environmental conditions (Catalá et al. 2017).

Hypsa et al. (2002) published the first molecular phylogeny that, among other goals, aimed on testing the monophyly of Triatominae. Based on several loci, this analysis indeed recovered Triatominae as monophyletic with low branch support (Fig. 1c); however, only two non-triatomine Reduviidae were included (one species each of *Reduvius* Linnaeus and *Arilus* Hahn), making it a relatively preliminary test of the monophyly of the group. Nevertheless, this result was corroborated using a morphological dataset and comprehensive sampling of Reduviidae, but only including four species of Triatominae (three Triatominae, one Rhodniini) (Weirauch 2008). Similar to Hypsa et al. (2002), this study also contributed little toward a rigorous test of the monophyly of Triatominae (Fig. 1d).

On a positive note and because of the extensive sample of predatory Reduviidae included, the study by Weirauch (2008) was the first to provide a meaningful hypothesis on putative close relatives of Triatominae. Triatominae were recovered in a clade together with the monophyletic Stenopodainae and several taxa of the polyphyletic Reduviinae. The triatomine sister taxon was represented by *Opisthacidius* Berg and *Centrogonus* Bergroth, two large-bodied reduviine taxa from the Neotropical and Australian regions, respectively, with *Zelurus* Burmeister and *Neivacoris* Lent & Wygodzinsky (both Neotropical) being more distantly related. Most subsequent molecular phylogenies included species of *Opisthacidius*, *Zelurus*, and/or Stenopodainae (but not the other two genera, nor additional large-bodied Reduviinae) and all have found typically high support for this clade sometimes referred to as the *Zelurus* clade of Reduviinae + Triatominae + Stenopodain ae (Georgieva et al. 2017; Hwang and Weirauch 2012; Justi et al. 2016; Patterson and Gaunt 2010; Weirauch and Munro 2009; Zhang et al. 2016a). In all analyses that included *Opisthacidius*, this genus forms either the sister taxon of Triatominae (Justi et al. 2016; Patterson and Gaunt 2010) (Figs. 1e, f) or renders Triatominae paraphyletic by being more closely related to either Rhodniini (Hwang and Weirauch 2012) or Triatomini (Zhang et al. 2016a) (Fig. 1g). *Zelurus* is often recovered as the sister lineage to *Opisthacidius* + Triatominae, with Stenopodainae being more distantly related. All but one of these hypotheses are based on small "legacy" or Sanger sequencing datasets; the phylogenomic analysis by Zhang et al. (2016) employed a hybrid transcriptome/Sanger strategy, but included transcriptome-level data for only three species in the entire clade (*Triatoma protracta* Uhler and two Stenopodainae). It appears evident that the way forward is to a) build phylogenomic datasets that provide sufficient phylogenetic signal to unequivocally resolve the monophyly/paraphyly issue of Triatominae and identify their sister lineage; and b) include in

these analyses additional taxa of Reduviinae that are suspected to be part of or closely related to the *Zelurus* clade.

Unfortunately, very little is known about the biology and ecology of Stenopodainae and *Zelurus* clade species (Ferreira et al. 2016; Lent and Wygodzinsky 1956; Weirauch et al. 2014). Stenopodainae have worldwide distribution with greatest diversity in the tropics of the Old and New Worlds. They comprise about 600 species in more than 110 genera, are typically brown with body shapes ranging from very elongate and stick-like to broad oval, and are diagnosed by their distinctive antennal morphology, among other features. Their earliest diverging lineage is restricted to the Old World tropics (Hwang and Weirauch 2012), but no formal biogeographic analysis has been conducted for the group. Some species have been beaten from trees or swept from grasses, but the majority have only been collected in light traps, suggesting that most have nocturnal activity patterns (Weirauch et al. 2014). Similarly, some species of the large Neotropical genus *Zelurus* (~130 spp.) are attracted to light traps, while others are actively flying diurnal predators (Haviland 1931), and yet others inhabit caves (Ferreira et al. 2016). *Opisthacidius* includes only eight described species with distributions ranging from Argentina to Mexico (Lent and Wygodzinsky 1956). Judging from specimen label information, personal observation, and online photo sharing sites, *Opisthacidius* spp. are mostly active at night. Intriguingly, a single female specimen of *Opisthacidius rubropictus* (Herrich-Schaeffer) was collected from the nest of a red-rumped cacique (Icteridae). This record appears to be the only microhabitat association reported in the literature but has been used as evidence that the most recent common ancestor of *Opisthacidius* and Triatominae may have been associated with the nest of vertebrates (Hwang and Weirauch 2012). This is in line with a previously proposed scenario by Schofield and colleagues that envisioned the evolution of hematophagous Triatominae as a transition from free-living to nest-dwelling predators and then to vertebrate feeding nest-dwellers (Schofield and Dujardin 1999). Focused research on the biology, ecology, and physiology of species of *Opisthacidius* and *Zelurus* and related groups is clearly critical to better understand the evolutionary transitions between predatory assassin bugs and hematophagous kissing bugs.

3 Evolutionary Relationships Within Triatominae

A steady number of phylogenetic analyses with focus on relationships within the Triatominae have been published during the past two decades (de Paula et al. 2007; Justi et al. 2014, 2016; Monteiro et al. 2000). Nevertheless, a substantial number of relationships have remained untested or are poorly supported. Importantly among these issues, published phylogenetic analyses have failed to include representative taxon sampling for the three small tribes of Triatominae. While both species of Cavernicolini are now represented in phylogenetic hypotheses (Georgieva et al. 2017), Alberproseniini lack from all published phylogenies, and *Microtriatoma trinidadensis* (Lent) is the only included species of Bolboderini. In addition, less

than a handful of analyses across Triatominae have included sufficient taxon sampling to meaningfully test and corroborate the monophyly of the two large tribes, Rhodniini and Triatomini (e.g., Justi et al. 2016), while other studies have focused on relationships at the genus and species group levels.

There is emerging evidence that both Bolboderini and Cavernicolini are more closely related to Rhodniini than they are to Triatomini. While *M. trinidadensis* and *Cavernicola pilosa* Barber were recovered as sister taxon to the Rhodniini in the analyses by Patterson and Gaunt (2010) and Hwang and Weirauch (2012), respectively, neither study sampled both species. Georgieva et al. (2017) included species of Cavernicolini (both *Cavernicola lenti* and *C. pilosa*) as well as Bolboderini (*M. trinidadensis*). Rhodniini were recovered with the highest branch support, but support for the clade comprising the three tribes was low (50%); even though Cavernicolini are shown as sister lineage to the remaining two tribes in their best likelihood tree, this relationship is not supported by bootstrap analyses and should be interpreted as a polytomy (Fig. 1h). It is now critical to include additional taxa of Bolboderini to further test this emerging set of relationships.

Within the Bolboderini, *Parabelminus* Lent shares a number of unique features with *Microtriatoma*, including the presence of a fossula spongiosa (tibial hairy attachment structure) on all three pairs of legs, suggesting that these two genera may be sister taxa (Lent and Wygodzinsky 1979). In their morphology-based diagram (Lent and Wygodzinsky 1979) indicated that *Belminus* may be the sister lineage to that clade, with *Bolbodera* outlined as the earliest diverging lineage. This scenario was tested by Gil-Santana (2014) employing a morphological dataset and cladistic approaches. In contrast to Lent and Wygodzinsky (1979), this analysis recovered *Belminus* and *Bolbodera* Valdes as sister clades, and *Microtriatoma* as sister taxon to all other Bolboderini. According to that analysis, the monophyly of Bolboderini is supported by several synapomorphies, including the elongate and distally detached maxillary plate and a spine-like projection on the antennifer (Gil-Santana 2014). This hypothesis will benefit from additional testing using molecular and morphological datasets.

4 Relationships Within Rhodniini

Rhodniini are well supported as a clade in molecular phylogenetic analyses and morphological characters long used to define the tribe likely represent synapomorphies, among them the postocular callosities, details of the male genitalia, and potentially also the number and position of pedicellar trichobothria (Lent and Wygodzinsky 1979). There is now overwhelming evidence that *Psammolestes* is derived from a *Rhodnius*-like ancestor and that *Psammolestes* renders *Rhodnius* paraphyletic. Specifically, the three species of *Psammolestes* typically form a well-supported clade and are recovered as sister lineage to the *prolixus* group within *Rhodnius* (de Paula et al. 2007; Georgieva et al. 2017; Justi and Galvão 2017). Relationships between this *prolixus* clade (Justi et al. 2016) and the two remaining

species groups of *Rhodnius,* the *pallescens* and *pictipes* groups, are less clear; although different studies recover both as monophyletic, either the *pallescens* (de Paula et al. 2007; Justi et al. 2016) or the *pictipes* groups (Georgieva et al. 2017) are recovered as sister lineage to all remaining Rhodniini, suggesting that taxa and data may need to be added to resolve this set of relationships. Many systematists agree that the most informative biological classifications are based on diagnosable monophyletic taxa, but there has been reluctance to update the classification of Rhodniini to reflect this.

5 Relationships Within Triatomini

Similar to the situation for Rhodniini, evidence that all extant Triatomini are derived from a common ancestor is now fairly strong (de Paula et al. 2005; Georgieva et al. 2017; Hypsa et al. 2002; Justi et al. 2014, 2016). This is in contrast to earlier scenarios that assumed Triatomini to be paraphyletic (Schofield and Galvão 2009). The three taxonomically most densely sampled and relatively data rich phylogenetic hypotheses (Georgieva et al. 2017; Justi et al. 2014, 2016) corroborate several relationships recovered in prior analyses based on more restricted sets of data and taxa. Among these results are the fairly well-supported monophyletic *dispar* group that forms the sister lineage to all remaining Triatomini; the monophyletic *infestans* group that comprises the bulk of the Neotropical species of *Triatoma*; and a clade that comprises species of *Panstrongylus* Berg, *Eratyrus* Stål, and *Linshcosteus* Distant, in addition to the Nearctic and northern Neotropical as well as Oriental species of *Triatoma*. Additional data and taxa will need to be included in analyses to stabilize relationships within the two larger of these clades.

6 Implications for the Evolution of Triatominae

Despite the instability in certain areas of the triatomine phylogeny and pending corroboration on their closest predatory relatives, current phylogenetic hypotheses together with morphological, biological, and ecological data layers are sufficient to formulate a number of hypotheses on the origin and evolution of Triatominae. The following paragraphs do not aim on outlining a comprehensive set of such hypotheses but highlight some of the interesting avenues for research that become feasible once robust phylogenies have been assembled.

The most recent common ancestor of Triatominae was likely fairly large-bodied. This can be deduced from the fact that species of *Opisthacidius* are large, as are the more distant relatives *Zelurus* spp. and the early diverging lineages of Stenopodainae, but the most recent common ancestor of Triatomini was also likely fairly large. In contrast, the Cavernicolini + Bolboderini + Rhodniini clade may have experienced

a reduction in body size during the early divergences and have reversed to larger body size within the *Rhodnius* + *Psammolestes* clade.

The transition to hematophagy occurred likely only once within the Reduviidae, in the most recent ancestor of all Triatominae. Early studies showed that the salivary protein composition differed between Rhodniini and Triatomini (Ribeiro et al. 1998). This was taken as evidence that hematophagy evolved separately in these two groups from predatory feeding strategies. However, such differences cannot be interpreted as independently derived without comparison with closely related predatory taxa. Comparative analyses of the sialotranscriptomes of Triatominae and their predatory relatives are still unavailable but offer the possibility of exciting insights into the evolution of the complex salivary compounds required to feed on vertebrate hosts. With regard to the morphological modifications involved in the transition from predatory behaviors to vertebrate blood-feeding, the maxillary and mandibular styles of Triatomini and Rhodniini share a unique set of characteristics not seen in other Reduviidae that are likely shared derived features (Barth 1954; Lent and Wygodzinsky 1979; Weirauch 2008; Wenk et al. 2010). Among them are the very slender tip of the mandible with one row of teeth (closely related groups have a flattened mandible with transverse ridges and teeth), the right maxillary stylet without rows of processes (occur in most predatory Reduviidae), and a valve formed between right and left maxillary stylets (Weirauch 2008). Similarly, the ability to flex the ultimate labial segment dorsad that is facilitated by a shift in the apodeme of the flexor muscles is not seen outside of Triatominae (Lent and Wygodzinsky 1979; Weirauch 2008).

It is unclear on which vertebrate species or group of species the most recent ancestor of all Triatominae may have fed upon. A recent study has reconstructed the common ancestor of Cimicidae based on phylogenetic data and ancestral state reconstruction and concluded that the most recent ancestor of bed bugs was likely associated with bats (Roth et al. 2019). A comparable analysis with focus on Triatominae has not been published to date. Also, such a reconstruction is likely less meaningful in Triatominae due to the high degree of polyphagy (feeding on more than one vertebrate host species or host clade) in this clade, together with the scarcity of reliable host association data from non-anthropogenic environments. Georgieva et al. (2017) assembled host data from the literature, supplemented with new DNA-based vertebrate host associations and visualized data for both Triatominae and vertebrate hosts plus insects (their Fig. 1). It appears evident from this diagram that there is no clear pattern, at least based on current phylogenetic hypotheses and available feeding data. Several of the early diverging lineages of Triatominae, namely species of Cavernicolini, Bolboderini, and the *dispar* clade of Triatomini have been found in association with bats and an ancestral association of Triatominae would be consistent with the "bat seeding" hypothesis that proposes that *Trypanosoma cruzi* Chagas evolved from bat-associated ancestors (Hamilton et al. 2012). However, Cavernicolini have also been observed with rodents, Bolboderini with mostly early diverging mammals, birds, and even reptiles and frogs, and species of the *dispar* group with various mammals and with birds

(Georgieva et al. 2017). Gut content of *Triatoma dispar*, the only species among these groups for which DNA-based host evidence is available, was identified as kinkajou (Procyonidae). Assuming that both the phylogenetic hypotheses and these feeding associations are correct, the most recent common ancestor of Triatominae likely fed rather opportunistically on a diverse range of vertebrate species.

Triatominae evolved likely much later than the other major lineage of hematophagous Heteroptera, the Cimicidae. The timing of the evolution of Triatominae has remained relatively poorly understood and has relied heavily on molecular phylogenies, using either fixed or relaxed molecular clocks based on fossil calibrations (Bargues et al. 2000; Hwang and Weirauch 2012; Justi et al. 2016; Patterson and Gaunt 2010). While early fixed-clock hypotheses dated the origin of Triatominae to between 64 and 49 mya (Bargues et al. 2000) and 110–107 mya (Patterson and Gaunt 2010), more recent estimates using relaxed clocks have converged on an Eocene or even Oligocene origin for the group (Hwang and Weirauch 2012; Justi et al. 2016). The congruence between the two latter hypotheses is unsurprising because they were based on the same set of fossil calibrations and similar sampling of non-triatomine Reduviidae; however, several of the fossils used to calibrate these phylogenies are suspected to require phylogenetic revision (Weirauch, unpublished), suggesting that these divergence dates may need to be reevaluated.

Fossils provide more direct evidence for the deep evolutionary history of higher groups by supplying definitive minimum ages. Unfortunately, the two fossil species that can be unambiguously attributed to the Triatominae, *Panstrongylus hispaniolae* Poinar, and *Triatoma dominica* Poinar (Poinar Jr. 2005, 2013) are too young to provide significant insights into the timing of deep divergences of Triatominae. The recently described mid-Cretaceous fossil *Triatoma metaxytaxa* Poinar (Poinar 2019), even though classified as Triatominae by the author, lacks diagnostic and synapomorphic features that would unambiguously associate this taxon with the Triatominae and it more likely represents one of the earlier diverging lineages of Higher Reduviidae.

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Taxonomy

Cleber Galvão

"The idea of good species … is a generality without foundation, an artifact of the procedures of taxonomy."

Ehrlich and Holm (1962)

Abstract The members of the subfamily Triatominae are true bugs specialized in blood-sucking. All species are potential vectors of *Trypanosoma cruzi* (Chagas, 1909), the causative agent of Chagas disease, although relatively few have epidemiological significance as vectors of the infection to the humans. The incidence of Chagas disease is declining, after the successful vector control campaign through Americas, but, the disease remains as a major problem to public health in Latin America. A few species of triatomines are found also in Asia and Oceania where the vector-borne transmission of *T. cruzi* does not occur. After Carlos Chagas discovered their importance as vectors, triatomine bugs have attracted permanent attention, and, thus, several aspects of their systematics, biology, ecology, biogeography, and evolution have been studied. Since the first species description, at the end of the eighteenth century, until the current approximately 150 extant species considered as valid, their classification remains mainly based in the traditional morphology. However, modern and diversified methods applied to studies of their characteristics, such as molecular approaches, are improving the systematics of these vectors. In the present chapter, the author summarizes the current knowledge on the taxonomy of the Triatominae.

Keywords Chagas disease · Taxonomy · Triatominae · Systematics

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1 Introduction

The blood-sucking insects of the subfamily Triatominae (Hemiptera, Heteroptera, Reduviidae) are vectors of Chagas disease, an infection caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Trypanosomatida, Trypanosomatidae) transmitted to humans and other mammals, primarily, through the feces of infected insects. All species are potential vectors of *T. cruzi* in the Americas, although relatively few have epidemiological significance as vectors of the infection to the humans. Triatomines are also found in Asia and Oceania, but, in these regions, the vector-borne transmission of *T. cruzi* does not occur, because the parasite is absent. Galvão and Justi (2015) summarized the information available about ecology, niches, its association with humans, and *T. cruzi* infection to all triatomine species. Triatominae is unusual within Reduviidae because all of its members are defined by their blood-sucking habit and show morphological adaptations associated with host-finding and feeding on vertebrate blood (Otálora-Luna et al. 2015). Since triatomines probably evolved from a predator ancestor, feeding on insects can be considered a primitive feature and the ancient triatomines probably fed on both insect and vertebrates (Weirauch and Munro 2009). This hypothesis could explain why some triatomine species remain able to feed on other invertebrates (Sandoval et al. 2004, 2010). Because of their importance as vectors, triatomine bugs have attracted permanent attention, and, thus, several aspects of their systematics, biology, ecology, biogeography, and evolution have been studied. Their classification remains mainly supported by morphology; however, the modern and diversified methods applied to studies of their characteristics, such as molecular approaches, are improving their systematics.

2 Historical Background

Mora et al. (2011) estimated that, there must be 8.7 million species considering all domains of life on Earth. These authors suggest that about 86% of the species on earth, and 91% in the ocean, still await description. Indeed, the number of species already described does not reach two million (May 1992; Wilson 1992). Systematics is the branch of biology in which the biodiversity is studied and identified, including the fields of classification and nomenclature. After the discovery of a supposed new species, a careful hypothesis should be proposed to discuss the combination of characteristics that differentiates the new species from a pre-existing one or others in the same group. The established taxonomic procedure may be time-consuming and occasionally tedious; however, only when the name and respective description of a new species are published and made available to the scientific community, it does formally exist or is considered as valid under the rules and concepts of the current five Biological Nomenclature Codes (Zoology, Botany, Bacteria, Viruses, and Cultivated plants).

In 1758, Linnaeus (1707–1778) published the tenth edition of *Systema Naturae*, in which he proposed rules for classifying and naming animals and plants, replacing the long denominations of species to hierarchical categories and the binomial nomenclature. The current nomenclatorial rules remain based in a traditional Linnaean frame in which the species concept is typological (primarily based in morphological characteristics), and a type specimen is the basis for defining the species (Mayr 1996; Ereshefsky 1997). After the publication in 1859 of "*On the Origin of Species by means of Natural Selection*" by Charles Darwin (1809–1882), the goal became to ensure that the groupings used to reflect a common evolutionary history, justifying classification as a reflection of genealogy, showing to us that the systematic hierarchy, until that moment, was only an approximation of evolutionary history (Mayr and Bock 2002).

Advancements in the classifications have promoted a lot of debate about the use of different sources of evidence for phylogenetic inferences. The idea of classifying species on the basis of molecular data emerged after the advent of molecular biology. The current feasibility for molecular analyzes showed the importance and reliability of molecular compared to morphological data. As far as it seems, molecular data are useful tools which make the exploration of biodiversity easier, while the morphological tools are at their limits (Asghar et al. 2015; Cameron 2014; Ebach 2011; Hughes and Piontkivska 2003; Stevens et al. 2011). Some authors comment that data sets from different sources should not be combined but analyzed separately since their independence would increase the significance of eventual corroboration. In this way, congruent trees obtained from independent data would provide the best estimate of the phylogeny. On the other hand, other authors defend the combined analysis, to formulate phylogenetic hypotheses based on all available information (Debruyne 2005; Page and Hughes 2011). Phylogenetic classifications became very robust, which led to the proposition of a specific nomenclatural code, the *PhyloCode*, a set of rules for naming clades and species and recommendations to phylogenetic nomenclature, differing from the current rank-based nomenclature codes (Cantino and Queiroz 2004). However, this code interferes not only in the application of names but also in the circumscription of the groups, as it does not allow to named paraphyletic and polyphyletic groups restricting your acceptance by taxonomists.

3 Taxonomy of the Triatominae, from De Geer to the DNA

3.1 The Beginning

In the tenth edition of the *Systema Naturae* included in "Classis V" (Insecta), "Ordo 2," Hemiptera, was the genus *Cimex* Linnaeus. The first Hemiptera, which would later be considered a triatomine, was described in this genus by De Geer (1773) as *Cimex rubro-fasciatus* (Fig. 1), from India, included by this author in his "third family of exotic bugs" ("Des Punaises exotiques of the troisieme famille"), curiously

Fig. 1 *Triatoma rubrofasciata* (De Geer, 1773), male, the first species of Triatominae described. Original generic combination and spelling of the specific name: *Cimex rubro-fasciatus*

this is the only species of Triatominae found in both the New World and Old World. The aspects and habits of a triatomine species were acknowledged a long time ago, since 1590, when a priest, Reginaldo de Lizárraga, while traveling to convents in Peru and Chile, noticed the presence of large hematophagous insects that attacked at night. In subsequent reports, other travelers and naturalists also mentioned the presence of these insects in South America, one of the most celebrated was a report by Charles Darwin, during his South American voyage aboard the HMS Beagle, in 1835 (Galvão 2003).

Thus, the beginning of the taxonomy of these vectors began with the publication: *Mémoire pour servir à l'histoire des insectes* (De Geer, 1773), where the first aforementioned species was described. Latreille (1807) created the subfamily Reduvini ("Réduvines") member of the family Cimicides. The group of "Hétéroptères" appeared in one of his subsequent works (Latreille, 1810). In 1811, the same author published: *Insectes de l'Amérique équinoxiale* describing *Reduvius dimidiatus* and *Reduvius geniculatus.* Laporte (1833) designated *Cimex rubro-fasciatus* as the type-species of the genus *Triatoma*, resulting in the current combination *Triatoma rubrofasciata*. Stål (1859) published the *Monographie der Gattung Conorhinus und Verwandten*, at the Berliner Entomologische Zeitschrift. In 1873a, b, Walker published the *Catalogue of the specimens of Hemiptera Heteroptera* in the *Collection of the British Museum,* divided in two parts (VII and VIII). Later, C. Berg, P.R. Uhler, G.C. Champion, G. Breddin, and W.L. Distant discovered several new species, resulting that until the beginning of the twentieth century more than 50 species were described. For more than one century, since the first description of De Geer (1773), triatomines were studied merely from a descriptive point of view. However, since

the discovery that they are actual or potential vectors of Chagas disease (Chagas 1909), the research involving them increased on several aspects. Neiva (1911) was one of the main contributors to the advance soon after the discovery of Carlos Chagas, describing several species in his thesis: "*Revisão do gênero* Triatoma *Lap.*" Important monographs were later published by Pinto (1925) and Del Ponte (1930), as well as other extensive works by Neiva and Lent (1936, 1941), Usinger (1944), Abalos and Wygodzinsky (1951), and Ryckman (1962) culminating with the most important systematic revision published by Lent and Wygodzinsky (1979).

3.2 Contributions to a Taxonomy Non-strictly Morphologic

The first attempt to use non-morphological characters to solve taxonomic issues is due to Actis et al. (1964, 1965) who used hemolymph protein electrophoresis to compare species of the *T. sordida* (Stål 1859) complexes. Similar but more comprehensive studies were published three years later by Brodie and Ryckman (1967). Since then, several studies using molecular markers for specific characterization or as a taxonomic tool have been published, revealing that, on the one hand, some species that are very similar morphologically may be genetically distinct, while on the other hand, species morphologically distinct may be related (Pérez et al. 1992, Panzera et al. 1996, Noireau et al. 1998, 1999, 2000a, b, 2002, Dujardin et al. 1999, Pavan and Monteiro 2007). The first phylogenetic trees built using molecular features were published by García and Powell (1998) and Stothard et al. (1998), followed by others as Lyman et al. (1999), Monteiro et al. (1999, 2002), Marcilla et al. (2002), Sainz et al. (2004), and de Paula et al. (2005, 2007). Unfortunately, most of these papers were based on small group of taxa and are hence unable to solve the questions of the entire subfamily phylogeny. Lent and Wygodzinsky (1979) defended the hypothesis that Triatominae is a monophyletic group pointing out three characters as possible autapomorphies of the subfamily: the hematophagous habit; the elongate and nearly straight labium with a flexible membranous connection between segments 3 and 4, allowing upwardly pointed distal rostral segment when the labium is in feeding position; and the loss of dorsal abdominal scent glands in nymphs. Schofield (1988) proposed an intuitive hypothesis to a polyphyletic origin to the triatomines. According with his view, the Asiatic fauna consists of two independent lineages derived from different reduviids. The first lineage consists of some species of *Triatoma* Laporte 1832, which had evolved from what was originally the New World species *T. rubrofasciata* after its introduction into the Old World. The second lineage, represented by the genus *Linshcosteus* Distant, 1904, would be a supposedly autochthonous Asiatic lineage of blood-feeding reduviids. This view was supported by Gorla et al. (1997) through morphometric analysis, where all species of *Linshcosteus* were shown to be unrelated to the species of *Triatoma* recorded from the Old World. This hypothesis was deconstructed by Hypsa et al. (2002) that conducted the first comprehensive molecular phylogenetic analysis of the subfamily, with the most representative sample (57 species) to study

the phylogeny and test the monophyly of the subfamily Triatominae. They included for the first time both New World and Old World species. Their results have led to the first formal proposition of taxonomic changes as reinclusion of *Linshcosteus* in Triatomini Jeannel, 1919; inclusion of the species of *Psammolestes* Bergroth, 1911 in *Rhodnius* Stål 1859; elevation of the *Triatoma flavida* Neiva, 1911 complex species to the genus *Nesotriatoma* Usinger 1944; inclusion of the *Triatoma spinolai* Porter, 1934 complex species in *Mepraia* Mazza, Gajardo & Jörg,1940, and inclusion of *T. dimidiata* (Latreille, 1811) in *Meccus* Stål 1859 (in the new combination, *M. dimidiatus*). From this point, attempting to solve this puzzle, several authors have used molecular data to infer phylogenetic relationships. Some phylogenetic reconstructions were published without a consensus about the monophyly, paraphyly, or polyphyly of the Triatominae. de Paula et al. (2005) using a molecular marker recovered Triatominae as polyphyletic in a study testing the sister status of the tribes Triatomini and Rhodniini Pinto, 1926 and also including several Reduviidae species. On the other hand, Weirauch (2008) using morphological traits of 21 subfamilies of Reduviidae supported the hypothesis of the subfamily Triatominae to be monophyletic, results which was corroborated by a molecular phylogeny of Reduviidae based on mitochondrial and nuclear ribosomal genes (Weirauch and Munro 2009). Hwang and Weirauch (2012), in a more extensive molecular analysis, reconstructed a phylogeny in which they observed the predatory reduviine genera *Opisthacidius* Berg, 1879 and *Zelurus* Hahn, 1826 to be closely related to Triatominae, recovered as paraphyletic. Justi et al. (2014) published molecular phylogeny of the tribe Triatomini, including 104 specimens from different populations of 54 species and 10 Rhodniini species, and a member of a distinct subfamily of Reduviidae (Stenopodainae) as the outgroup. Their results showed that the *Rhodnius prolixus* and *R. pictipes* groups were more closely related to each other than to the *R. pallescens* group. For Triatomini, they demonstrated that the complexes within the paraphyletic *Triatoma* genus were related to their geographical distribution. Additionally, they observed that the divergence within the *T. spinolai* and *T. flavida* complex was higher than in the other *Triatoma* complexes, proposing that these complexes should be ranked under the genera *Mepraia* and *Nesotriatoma*. Finally, these authors suggested a morphological investigation of the paraphyletic genera *Triatoma* and *Panstrongylus* Berg, 1879. Despite the efforts to elucidate the evolution of the Triatominae, further studies including higher diversity of the species, more approaches and analyses are fundamental to solve this question. An updated and extensive revision on the evolution and phylogenetic relationships of the Triatominae was provided by Monteiro et al. (2018).

The morphometry, another tool applied to taxonomy of triatomines, appeared in the 1990s of last century, as an attempt to contribute to the conventional analyses. Later, as a refinement of this method, the geometric morphometry, also began to be applied in triatomine studies, revolutionizing the quantification and analysis of morphological variation. Geometric morphometry allows for the accurate estimation and separates analytic assessment of the size and shape of phenotypic traits, and the way they vary (Rohlf and Marcus 1993). According to some authors geometric morphometric analyses could be a tool for the study of taxonomic difficulties within the

Triatominae (Matías et al. 2001; Villegas et al. 2002; Dujardin et al. 2009; Gurgel-Goncalves et al. 2011), as well as to ontogenetic studies of immature forms (Galvão et al. 2005; Rocha et al. 2005). Recently, the possibility of automating of the identification of triatomines has been developed through a fully automated visual identification system (Gurgel-Gonçalves et al. 2017; Khalighifar et al. 2019).

Following the comprehensive revision of the subfamily Triatominae by Lent and Wygodzisnky (1979), some systematic problems still remain, especially at genus and species levels. The classification based on morphological traits has been challenged by new arrangements based on molecular data. After the revision by Lent and Wygodzisnky (1979), a publication by Galvão et al. (2003) has provided useful updates of new taxa and taxonomic changes, sorted the known species in 19 genera. Among the latter, however, the genus *Torrealbaia* Carcavallo, Jurberg & Lent, 1998 was shown to actually belong to Harpactorinae and was synonymized with *Amphibolus* Klug, 1830 by Forero et al. (2004). Later, Schofield and Galvão (2009) reorganized the genus *Triatoma* into three groups and eight complexes, suggesting the synonym of the genera *Meccus*, *Mepraia*, and *Nesotriatoma* under *Triatoma* in an attempt to propose a pragmatic taxonomy. Currently, Triatominae consists of 153 extant and 3 fossil species assigned to five tribes and 18 genera (Table 1) (Justi and Galvão 2017, Dorn et al. 2018, Oliveira et al. 2018, Lima-Cordon et al. 2019, Nascimento et al. 2019, Ponair Jr. 2019; Alevi et al. 2020; Zhao et al. 2021).

4 Classification

4.1 Hemiptera-Heteroptera (Truebugs)

Considered the largest group of hemimetabolous insects, with more than 42,000 described species in about 90 families (Henry 2009), the true bugs are widely distributed and greatly diversified in tropical zones. The order suborder Heteroptera of the order Hemiptera is divided into the following infraorders as recognized by Schuh (1979): Enicocephalomorpha, Dipsocoromorpha, Gerromorpha, Nepomorpha, Leptopodomorpha, Cimicomorpha, and Pentatomomorpha. The forewings are one of the main characteristics of the majority of Heteroptera, which presents a thickened and leathery area from the anterior part to the middle (corium) and a membranaceous area from the middle to the distal part (Weirauch and Schuh 2011). This kind of forewing is named hemelytron (plural hemelytra) which gave the name to the order Hemiptera (Hemi = half, ptera = wing) (Linnæus 1758). It is noteworthy that, currently, other suborders are included in Hemiptera (such as Auchenorrhyncha and Sternorrhyncha), in which the forewing is roughly uniform and does not present the aforementioned characteristics of the hemelytra of the suborder Heteroptera. The piercing-sucking apparatus of the heteropterous is characterized by four piercing stylets homologous with the mandible and maxillae of the basic chewing mouthparts (Cobben 1978). Most species are terrestrial and some are

Table 1 Current taxa classification of the Triatominae

Subfamily	Tribes	Genera	Living species		Fossil species	
Triatominae	Alberprosenini	*Alberprosenia*	2	*goyovargasi*, *malheiroi*		
	Bolboderini	*Belminus*	8	*corredori*, *costaricensis*, *ferroae*, *herreri*, *laportei*, *peruvianus*, *pittieri*, *rugulosus*		
		Bolbodera	1	*scabrosa*		
		Microtriatoma	2	*borbai*, *trinidadensis*		
		Parabelminus	2	*carioca*, *yurupucu*		
	Cavernicolini	*Cavernicola*	2	*lenti*, *pilosa*		
	Rhodniini	*Psammolestes*	3	*arthuri*, *coreodes*, *tertius*		
		Rhodnius	21	*amazonicus*, *barretti*, *brethesi*, *colombiensis*, *dalessandroi*, *domesticus*, *ecuadoriensis*, *marabaensis, micki, milesi*, *montenegrensis, nasutus*, *neglectus*, *neivai*, *pallescens*, *paraensis*, *pictipes*, *prolixus*, *robustus*, *stali*, *zeledoni*		
	Triatomini	*Dipetalogaster*	1	*maxima*		
		Eratyrus	2	*cuspidatus*, *mucronatus*		
		Hermanlentia	1	*matsunoi*		
		Linshcosteus	6	*carnifex, chota, confumus, costalis, kali, karupus*		
		Mepraia	3	*gajardoi, parapatrica, spinolai,*		
		Nesotriatoma	3	*confusa, flavida*, *obscura*		
		Panstrongylus	14	*chinai*, *diasi*, *geniculatus*, *guentheri*, *howardi*, *humeralis*, *lenti*, *lignarius*, *lutzi*, *martinezorum*, *megistus*, *mitarakaensis*, *rufotuberculatus*, *tupynambai*	1	*hispaniolae*
		Paratriatoma	1	*hirsuta*		

(continued)

Table 1 (continued)

Subfamily	Tribes	Genera	Living species		Fossil species	
		Triatoma	75	*T. amicitiae, arthurneivai, bahiensis, baratai, barberi, bolivari, boliviana, bouvieri, brailovskyi, brasiliensis (includes two subspecies, the nominotypical and macromelasoma), breyeri, carcavalloi, carrioni, cavernicola, circummaculata, costalimai, deaneorum, delpontei, dimidiata, dispar, eratyrusiformis, garciabesi, gerstaeckeri, gomeznunezi, guasayana, guazu, hegneri, huehuetenanguensis, incrassata, indictiva, infestans, jatai, juazeirensis, jurbergi, klugi, lecticularia, lenti, leopoldi, limai, maculata, matogrossensis, melanica, melanocephala, mexicana, migrans, mopan, neotomae, nigromaculata, nitida, oliveirai, patagonica, peninsularis, petrocchiae, pintodiasi, platensis, protracta, pseudomaculata, pugasi, recurva, rosai, rubida, rubrofasciata, rubrovaria, ryckmani, sanguisuga, sherlocki, sinaloensis, sinica, sordida, tibiamaculata, vandae, venosa, vitticeps, williami, wygodzinskyi*	1	*dominicana*
		T. phyllosoma complex (=Meccus)	6	*bassolsae, longipennis, mazzottii, pallidipennis, phyllosoma, picturata*		
		Paleotriatoma †			1	*metaxytaxa*
Total	**5**	**18**	**153**		**3**	

†= fossil genus

Based on Galvão and de Paula (2014), Bargues et al. (2017), Justi and Galvão (2017), Dorn et al. (2018), Oliveira et al. (2018), Lima-Cordon et al. (2019), Nascimento et al. (2019), Ponair Jr. (2019), Alevi et al. (2020), Zhao et al. (2021)

aquatic, many suck plants, others are predators or entomophagous, and all species of the subfamily Triatominae are hematophagous. Even though the hematophagous habits evolved independently, only a few members of the suborder Heteroptera suck blood. Cimicidae (including bed bugs) feed exclusively on vertebrate's blood, usually of birds, bats, and humans, Polyctenidae (bat ectoparasites) also feed exclusively on bats and some species of the tribe Cleradini (Hemiptera, Lygaeidae, Rhyparochrominae) are at least facultative blood-suckers (Harrington 1988; Schuh and Slater 1995; Otálora-Luna et al. 2015). Other eventual records could reflect accidental feeding (Schaefer 2000, 2004). In a recent paper about habitat and lifestyle in Heteroptera, Weirauch et al. (2019) used combined morphological and molecular phylogeny analyses to demonstrate a converged and well-supported hypothesis of heteropteran infraordinal relationships. Moreover, their results suggested that aquatic and semi-aquatic true bugs invaded these habitats three times independently from terrestrial habitats.

4.2 Reduviidae Latreille, 1807 (Assassin Bugs)

The family Reduviidae (Hemiptera: Heteroptera), so-called assassin bugs, is one of the most diverse groups of true bugs that exhibit predatory or hematophagous feeding habits and show a great morphological diversity. Reduviidae range from delicate and elongate to large and robust or ovoid body shapes. Some of the most distinctive characteristics of the assassin bugs are the necklike shape of the head behind the eyes and the labium, which is short, generally strongly curved (sometimes straight), inflexible and with three visible segments in most subfamilies (four in two subfamilies only). Other relevant body structures include the prosternum with a stridulatory groove (stridulitrum); membrane of hemelytra, usually with two or three elongated cells; the presence of a fossula spongiosa at the apex of the fore and mid tibiae in many taxa; and the presence of Brindley's glands between the metathorax and the first abdominal segment. The internal female genitalia has lateral spermathecae; males with the eighth abdominal segment telescoped largely into the seventh segment and usually with symmetrical genitalia (Schuh and Slater 1995; Weirauch 2008).

4.3 Triatominae Jeannel, 1919 (Kissing Bugs; Cone-Nose Bugs)

While the members of most Reduviidae subfamilies feed on invertebrates, those of the subfamily Triatominae are obligatory hematophagous in all phases of their development, feeding across a broad range of mammal and other vertebrate species, although there are some species able to feed on invertebrates (kleptohematophagy and hemolymphagy) and by coprophagy (Sandoval et al. 2000, 2004, 2010). In

general, most species are nocturnal, and during the day they remain in their resting places, although they may sometimes go out to suck blood during the day under adverse conditions. Schofield (2000) suggested that the transition from predatory to hematophagous lifestyles occur several times within Triatominae. However, this assumption remains questionable. A detailed revision on the evolution of hematophagous habits in Triatominae was published by Otálora-Luna et al. (2015).

Within Triatominae, characteristics commonly used to distinguish genera and species include the general color of the body and legs, and morphological aspects of the head and pronotum. Most species can be easily identified on the basis of their morphological characteristics, (Lent and Wygodzinsky 1979), only a few species in the so-called complexes or sibling species needs molecular, cytogenetic, and morphometric tools for the clarifying their specific status. The length of species varies from approximately 5 mm in *Alberprosenia goyovargasi* Martínez & Carcavallo, 1977, to approximately 44 mm, in *Dipetalogaster maxima* (Uhler, 1894) and the color pattern varies, with an overall black or piceous color and spotted patterns of yellow, brown, orange, or red (Fig. 2).

The male external genitalia is composed of approximately 15 structures highly variable, therefore, useful to generic and specific differentiation. The female external genitalia was described for most species of the subfamily (Lent 1948; Abalos and Wygodzinsky 1951; Sherlock and Serafim 1967), but their diagnostic importance was denied in all papers published by Lent and Jurberg (1968, 1969, 1975) which considered them uniform, therefore, not useful for specific identification. The resurrection of female genitalia, as an important taxonomic tool, was attributed to Rosa et al. (2010) through a detailed study by scanning electron microscopy. Subsequently, several studies corroborate the diagnostic value of female genitalia (Rosa et al. 2012, 2014, 2017; Rodrigues et al. 2018).

Fig. 2 Coloration pattern of triatomine species (live specimens): (**a**) *Panstrongylus megistus*, (**b**) *Rhodnius stali,* (**c**) *Triatoma tibiamaculata*

The nymphs differ from adults by the smaller eyes, the absence of ocelli, of hemelytra and hind wings, as well as of external genitalia. Their pronotum is not shield-like, the tarsi have invariably two segments (three in all adult triatomines except *Microtriatoma and Parabelminus*), there are no spongy fossa (except in *Microtriatoma* and *Parabelminus*), and the sclerotization of the abdomen is incomplete (Lent and Wygodzinsky 1979). The morphology of triatomine eggs and nymphs has been studied by several authors, a summary of these works was provided by Galvão (2014).

4.4 Tribes and Genera

Triatomini Jeannel, 1919 (the Most Speciose Tribe)

Triatomini is the most diverse tribe, including more than 70% species of the subfamily, the genus *Triatoma* is the most speciose within the tribe (75 species), followed by *Panstrongylus* (14 species). The tribe has the widest geographical distribution among Triatominae reaching an extensive range of ecotopes. The high morphological diversity of *Triatoma* has led Schofield and Galvão (2009), as an attempt to rearrange the genus, to divide it into three groups, eight complexes, two of which (*T. phyllosoma and T. infestans*), divided into eight subcomplexes, based on both morphological traits and geographical distribution. However, it is noteworthy that groups and specific complexes are not formally recognized as taxonomic entities. This diversity reflects a complex evolutionary history of the tribe, considered a paraphyletic group; indeed, the paraphyly of *Triatoma* with respect to the other genera as *Dipetalogaster*, *Eratyrus*, *Linshcosteus Mepraia Panstrongylus*, *Paratriatoma* was showed in several systematic studies (Hypsa et al. 2002, Marcilla et al. 2002, Hwang and Weirauch 2012, Justi et al. 2014, Ibarra-Cerdena et al. 2014).

***Triatoma* Laporte 1832**. The genus *Triatoma* was named based on specimens with broken antennae showing only three of the four antennal segments (*Triatoma* meaning three antennal segments). On examination of fresh specimens, and realizing his mistake, he changed the generic name to *Conorhinus* (Laporte 1832/33). However, the generic name *Triatoma* has nomenclatural priority and remains valid. Some studies have demonstrated taxa englobing cryptic species, which are being described more frequently (Monteiro et al. 2013). Two of the most recently described species, *T. mopan* Dorn, Justi & Dale, 2018 and *T. huehuetenanguensis* Lima-Cordón & Justi, 2019 are both closely related to *T. dimidiata* (Latreille, 1811). On the other hand, some supposed species described as new may be no more than variants, based on minor morphological differences, many of which might be progressively synonymized (Schofield and Galvão 2009). The variants can also arise through *morphological plasticity* where closely populations, after isolation, can display different phenotypes within a very few generations (Dujardin et al. 1999). From a phylogenetic point of view based mainly on nuclear or mitochondrial gene fragments, most of the *Triatoma* species (with a few exceptions) are clustered in two

main clades consistent with the geographical distribution: *Triatoma* of Central and North America and *Triatoma* of South America. A detailed framework of the phylogeny of *Triatoma* has been provided by Monteiro et al. (2018).

***Panstrongylus* Berg, 1879.** The genus was created with the description of the type species, *P. guentheri* Berg 1879. Species currently included in *Panstrongylus* and described before its creation were firstly included in the genus *Lamus* Stål 1859. *Lamus* was described to the species *L. megistus* and *L. geniculatus*, based on shape head and antennal insertion near to the eyes. However, because *Lamus* Stål 1859 was preoccupied by *Lamus* Stål, 1854, a genus of Pentatomidae, Kirkaldy (1904) create a new name for *Lamus* Stål 1859: *Mestor* Kirkaldy 1904. The latter was synonymized with *Panstrongylus* by Abalos and Wygodzinsky (1951). A few important taxonomic changes were proposed within *Panstrongylus*. *Panstrongylus herreri* and *P. lignarius* have been synonymized on the basis of ITS-2 rDNA sequences by Marcilla et al. (2002) and corroborated by cytogenetic similarity (Crossa et al. 2002). Garcia et al. (2005) studied *P. lutzi* (Neiva and Pinto, 1923) captured in the Brazilian state of Minas Gerais and found that it may show intraspecific variations in its phallic structures compatible with the description of *P. sherlocki* Jurberg et al. 2001. The two last described species, *P. mitarakaensis* Bérenger & Blanchet, 2007 and *P. martinezorum* Ayala, 2009 looks to be closely related to *P. geniculatus.* Patterson et al. (2009) compared the differences in head shape between *Triatoma* and *Panstrongylus* by morphometric analysis of fifth instar nymphs and adults of *P. megistus*, *T. lecticularia* (Stål 1859), *T. infestans* (Klug, 1834), and *Rhodnius prolixus* Stål 1859. Their results showed an overlap between the shape of the head of nymphs of *T. lecticularia* and *P. megistus*.

***Dipetalogaster* Usinger, 1939.** A monotypic genus, which the only species, *Dipetalogaster maxima* is the largest triatomine known (the female may attain 44 mm length). This species could be differentiated from all other triatomines by its extraordinarily large size, by a pleated abdomen and by the double invaginated "flask-like" organ on the third rostral segment. Its geographical distribution is restricted to the southern area of Baja California Sur, Mexico, living in dry rocky areas of the semi-desert region where its large size allows it to store an amount of blood to survive in fasting. It is an aggressive biter, attacking when hungry, feeding on any available vertebrate, including humans even under the daylight (Marsden et al. 1979).

***Eratyrus* Stål 1859**. The genus *Eratyrus* comprises only two species, *E. cuspidatus* and *E. mucronatus*, both species are considered sylvatic and potential vectors of *T. cruzi*, but studies about their biology and epidemiology are scarce. They can be differentiated from *Triatoma* by the unusually long antennae. The geographical distribution of *E. cuspidatus* is Central America and South America west of the Andes, while *E. mucronatus* occurs in the vast areas of South America (Lent and Wygodzinsky 1979).

***Hermanlentia* Jurberg & Galvão, 1997**. The only one species, *Hermanlentia matsunoi* (Fernández-Loayza, 1989), is known from cave habitats in the Peruvian Andes (Cuba Cuba et al. 2002), was first described as belonging to *Triatoma*.

Posteriorly, Jurberg and Galvão (1997), based on some differences in the male genitalia traits, reassigned it to a new monotypic genus.

***Linshcosteus* Distant, 1904.** The genus is composed of six closely related species, all restricted to continental India where they are found in various localities, usually occupying rockpiles associated with small rodents and bats (Patterson et al. 2001, Galvão et al. 2002). Until the 1970s of last century *L. carnifex* was the only known species, when *L. confumus* and *L. costalis* were described by Ghauri (1976). Three years later, Lent and Wygodzinsky (1979) described *L. chota* and *L. kali*. The sixth species, *L. karupus*, was described by Galvão et al. (2002). The genus is well characterized and can be easily differentiated from other Triatominae by the flattened body, unusually broad abdomen, absence of functional stridulatory sulcus and by a short labium, not reaching the proesternum (Lent and Wygodzinsky 1979, Galvão et al. 2002). Carcavallo et al. (2000) established the tribe Linshcosteusini for this genus; however, the morphological similarity suggested that *Linshcosteus* is less similar to the other triatomine genera than any one of them is to the others (Schaefer & Coscarón 2001) besides molecular data showed *Linshcosteus* and *T. rubrofasciata* are sister groups, therefore the tribe was considered invalid (Hypsa et al. 2002, Justi et al. 2014).

***Meccus* Stål 1859 (=*T. phyllosoma* complex)**. The genus *Meccus* was originally proposed by Stål 1859 for some members of the *T. phyllosoma* complex (including *T. phyllosoma*, *T. picturata*, *T. mazzotiii*, *T. longipennis*, and *T. pallidipennis*). It was subsequently reduced to subspecific rank by Usinger (1944). Later, *T. bassolsae* was described by Aguilar et al. (1999) into this group of six large Mexican species with the unusually wide abdomen and conspicuous thoracic tubercles. Lent and Wygozinsky (1979) recognizing a close relationship and unique traits of these species treated them as valid species, grouped into *T. phyllosoma* complex. The genus *Meccus* was revalidated by Carcavallo et al. (2000) based on morphological traits, a proposal posteriorly reinforced by molecular data obtained by Hypsa et al. (2002). The latter work, however, included only three of the six species that have been previously assigned to the *T. phyllosoma* complex. Martinez-Hernandez et al. (2010) raised again the hypothesis that these species are only morphotypes with chromatic and genetic varieties, which should be considered as subspecies. Finally, Justi and Galvão (2017) suggested that all species of *Meccus* should be grouped again into *Triatoma.*

***Mepraia* Mazza, Gajardo & Jörg, 1940.** The genus is composed of three species occurring in distinct regions of Chile. The generic names, *Mepraia* Mazza, Gajardo Tobar and Jorg 1940, and *Triatomaptera* Neiva and Lent, 1940, have been almost simultaneously proposed to the species *Triatoma spinolai* Porter, 1934 because of the remarkable alar polymorphism, unique in the subfamily Triatominae. *Triatoma chilena* Usinger (1939) and *Triatomaptera porteri* Neiva and Lent, 1940 were synonymized with *Triatoma spinolai* by Lent and Wygodzinsky (1979). The genus *Mepraia* was re-erected many years after by Lent et al. (1994) based mainly on male genitalia characters. Posteriorly more two species were described in this genus, *M. gajardoi* Frías-Lassere, Henry and González 1998 and *M. parapatrica* Frías-Lassere 2010. The females of the three species are micropterous while the

males of *M. gajardoi* are brachypterous, males of *M. parapatrica* can be brachypterous or macropterous and those of *M. spinolai* can be micropterous, brachypterous, or macropterous.

***Nesotriatoma* Usinger 1944.** Neiva (1911) described *Triatoma flavida* from Cuba. Later, Usinger (1944) proposed the generic name *Nesotriatoma*, upon the description of *N. bruneri* based on a female specimen from Cuba, including *T. flavida* in the new genus. After observations of morphometric variations in a series of specimens, Usinger (1946) synonymized *N. flavida* and *N. bruneri.* Maldonado (1962) added a new species to the genus, *N. obscura*. Lent and Wygodzinsky (1979) synonymized *Nesotriatoma* with *Triatoma*, transferring both species for the latter genus Posterior analyses of male genitalia led to the revalidation of *T. bruneri* by Lent and Jurberg (1981), Hypsa et al. (2002) included in their phylogenetic study, specimens identified as *T. bruneri* and specimens of *T. flavida*. Both species were recovered as monophyletic within the same clade with *Panstrongylus*, demonstrating, for the first time, the close relationship of *Triatoma* with *Panstrongylus,* leading the authors to propose the revalidation of the genus *Nesotriatoma* as an Antillean clade. González and Broche (2006) in their revision of the subfamily in Cuba, maintained the generic status of *Nesotriatoma* as well as Justi et al. (2014) also emphasizing the closer relationship with *Panstrongylus* than to the most species of *Triatoma*. Oliveira et al. (2018) examinated the type specimens of *T. flavida, N. bruneri* and the specimens examined by Lent and Jurberg (1981) in their revalidation of *N. bruneri.* These authors showed that the specimens used by Lent and Jurberg (1981) did not correspond to *N. bruneri,* but to a new species that they described as *Nesotriatoma confusa*, as a reference to the confusion that occurred in the description and revalidation of the species of the genus *Nesotriatoma*.

***Paratriatoma* Barber, 1938.** The only species, *Paratriatoma hirsuta*, occurs in Southwestern USA and Northwestern Mexico, associated with woodrats (*Neotoma* spp.) (Lent and Wygodzinsky 1979).

Rhodniini Pinto, 1926 (Genera Well Characterized, Species Cryptics)

The tribe Rhodniini contains two genera, ***Rhodnius*** Stål 1859 (with 21 species) and ***Psammolestes*** Bergroth, 1911, (with three species), despite their different morphologies and ecological habits, both are arboricolous, *Rhodnius* species live in tree crowns (especially palm trees) and *Psammolestes* strictly associated with the dendrocolaptid or furnariid bird's nests. The geographical distribution of *P. arthuri* is restricted to Colombia and Venezuela and north of the Amazon region, while *P. coreodes* and *P. tertius* are distributed throughout the Caatinga and Cerrado-Chaco. The geographical distribution of *Rhodnius* is wider; their species are distributed throughout the Amazon region, into the plains to the North, and the Caatinga and Cerrado to the South. Both genera are well characterized and can be easily differentiated from other triatomines. The main characters to distinguish *Rhodnius* and *Psammolestes* from the other genera are the apically inserted antennae and the presence of distinct callosities behind the eyes (Lent and Wygodzinsky 1979). On the

other hand, the species belonging to each genus are difficult to differentiate. The tribe seems to represent a monophyletic group, with morphological and genetic traits that distinguish them from other tribes. *Rhodnius* is divided into two lineages, one of them with two subgroups, the first lineage comprises the species of *R. prolixus-R. robustus* (*barretti*, *dalessandroi, domesticus*, *milesi*, *marabaensis, montenegrensis*, *nasutus*, *neglectus*, *neivai*, *prolixus*, *robustus*), and the second lineage including species of subgroup *R. pictipes* (*amazonicus*, *brethesi*, *paraensis*, *pictipes*, *stali*, *zeledoni*) distributed east of the Andes, and of the subgroup *R. pallescens* (*colombiensis*, *ecuadoriensis*, *pallescens*) distributed to the west of the Andes (Abad-Franch et al. 2009, Just and Galvão 2017). The genus *Psammolestes* was included in the *Rhodnius* clade by Hypsa et al. (2002), as "aberrant species of *Rhodnius*" corroborating the previous results that make both *Triatoma* and *Rhodnius* paraphyletic (Lyman et al. 1999). Recent research on the evolution of the Rhodniini showed to us that they are more closely related to Bolboderini and Cavernicolini than to the Triatomini (Hwang and Weirauch 2012). An extensive debate about the evolutionary history of the Rhodniini was published by Monteiro et al. (2018). Some species of *Rhodnius* deserves further studies to elucidate their taxonomic status. This is the case of *R. zeledoni* Jurberg, Rocha & Galvão, 2009, which seems to be very similar to *R. domesticus* Neiva and Pinto, 1923. The type specimen was found, very damaged, in Sergipe state, Brazil, region included in the distribution range of *R. domesticus*. Therefore, examination of further material is essential to confirm or not if *R. zeledoni* is a valid species. Following Monteiro et al. (2018), *R. marabaensis* Souza et al. 2016 could be no more than a variant, genetically very close to *R. robustus*. The recently described *R. taquarussuensis* Rosa et al. (2017) was synonymized with *R. neglectus* Lent, 1954 by Nascimento et al. (2019) based on interspecific crosses and molecular markers. They concluded that *R. taquarussuensis* is a phenotypic form of *R. neglectus* instead of a distinct species.

Bolboderini Usinger 1944 (Small Genera)

The tribe Bolboderini Usinger 1944 has been considered a monophyletic group, of small triatomines (adults up to 12 mm in length) divided into four genera, ***Bolbodera*** Valdés, 1910; ***Belminus*** Stål 1859; ***Microtriatoma*** Prosen & Martínez, 1952; and ***Parabelminus*** Lent, 1943 (Lent and Wygodzinsky 1979). *Microtriatoma* Prosen & Martinez, 1952 is a well characterized genus and can be easily differentiated from other triatomine. The basic color of its species is black with various body regions covered by distinct adpressed, short setae; strongly flattened bodies; adults measuring 7–8 mm in length. According to Lent and Wygodzinsky (1979) *Microtriatoma* could be considered to be the more plesiomorphic Bolboderini genus by the absence of denticles on the femora and the overall small size. Another interesting characteristic is the presence of three pairs of spongy fossae in all nymphal instars of *Microtriatoma* (and *Parabelminus*) a condition unique in the subfamily. This genus was described based on specimens of *M. trinidadensis* (Lent, 1951) by Prosen & Martínez (1952) (as *Microtriatoma mansosotoi*). However, Lent and Wygodzinsky

(1979) established the synonym of both specific names. Molecular data showed *Microtriatoma trinidadensis* as a basal taxon in relation to the Rhodniini (Patterson and Gaunt 2010). *Belminus* is the most speciose genus of Bolboderini; was described by Stål (1859) based on a single species, *Belminus rugulosus*, from Colombia. The genus comprises eight valid species which occur in Central America, Colombia, Peru, Venezuela, and northern Brazil (Sandoval et al. 2010). *Belminus* can be differentiated from other triatomine bugs by very small length and by head long, elongate, and fusiform (Sandoval et al. 2007). In addition to the original descriptions, taxonomic knowledge on the genus is restricted to a few morphological papers (Osuna and Ayala 1993; Rocha et al. 2005; Galvão and Angulo 2006; Sandoval et al. 2007; Gil-Santana and Galvão 2013) reflecting the fact that this is the one of the lesser known Triatominae genera. *Bolbodera* is a monospecific genus known only from Cuba.

Cavernicolini Usinger 1944 (Small Triatomines, Cave Specialized)

The monotypic tribe Cavernicolini differs from the typical Triatomini by the size of the ocelli and their position in relation to the post-ocular suture (Usinger 1944). It shares with the Rhodniini and Bolboderini, as well as, with the Triatomini genus *Paratriatoma* a membrane connecting the dorsal and ventral abdominal margins, and similarly with some of the Bolboderini, presents reduced ocelli. With only one genus, ***Cavernicola*** Barber 1937 and two species, they are considered cave specialized triatomines. *Cavernicola pilosa* was described from seven adult specimens and five nymphs collected in caves occupied by large numbers of bats in Panama (Barber 1937). The second species of the genus, *C. lenti* was described from adults, nymphs, and eggs collected inside a large live hollow tree in Amazonas State, Brazil, associated with *Rhipidomys* sp. (Rodentia), but are able to feed from other vertebrates in laboratory (Barrett and Arias 1985). Considering that the diagnoses of the tribe Cavernicolini and the genus *Cavernicola* were made before the description of the second species, Oliveira et al. (2007) redescribed the genus *Cavernicola* and the tribe Cavernicolini based in morphological and morphometric features.

Alberproseniini Martínez and Carcavallo, 1977 (the Smallest Triatomine)

The genus ***Alberprosenia*** Martínez & Carcavallo, 1977 has only two unusual small, triatomines species. *Alberprosenia goyovargasi* Martínez & Carcavallo, 1977 has only been collected in the Maracaibo basin dry forests, Venezuela, while *Alberprosenia malheiroi* Serra, Atzingen & Serra 1987 was captured in a hollow trunk of palm-tree associated with bats or birds, in the State of Pará, Brazil. Both species have been reared in the laboratory (Martínez and Carcavallo 1977; Carcavallo et al. 1995).

5 Conclusions

Throughout history, different concepts of taxonomy, systematics, and classification have been endorsed by several authors (Mayr 1981, 1996; Mayr and Bock 2002), with the theoretical and practical advances made since the publication of the *Systema Naturae*, the taxonomy will be beyond of the initial discovery of new species, evolving to more complex classification systems, incorporating concepts of Darwinian ideas on evolution and formation of species and Hennigian cladistics. These new approaches to the taxonomy of Triatominae are still young, despite the efforts to elucidate the evolution of the Triatominae, more approaches and analyses are fundamental to solve this question. For instance, the genome sequencing is in the beginning, this way, the scenario of the taxonomic studies and future discoveries are promising. Despite this captivating academic challenge, it is necessary to have in mind the usefulness of a pragmatic classification, maintaining caution, as much as possible, in taxonomic adjustments to facilitate the customary practice especially in the vector control activities.

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Speciation Processes in Triatominae

Marcio G. Pavan, Cristiano Lazoski, and Fernando A. Monteiro

Abstract This chapter intends to familiarize the reader with the basic concepts regarding speciation in insects, through the description and exemplification of the three most common speciation modes described in the specialized literature on the subject: the allopatric, parapatric, and sympatric speciation modes.

We also argue that nowadays there is, perhaps, an excess of species concepts to choose from. Two of those have been used more often by the Triatominae research community: the biological species concept and the phylogenetic species concept. The idea first advanced by De Queiroz (Syst Biol 56(6):879–886, 2007) that the proposition of a single species concept that would unify all concepts available is not only desirable but also essential at this point. The issue of overconservative systematics is considered with emphasis on the paraphyly of *Triatoma.* The implications of phenotypic plasticity in traditional triatomine taxonomy are also addressed.

How long does it take for a new species of triatomine to be formed? Early proposals envisioned very short time intervals say, a few hundred years, for the process to be completed. Two well-studied examples are presented.

How do triatomines speciate? Vicariance and allopatric speciation seem to be the norm in Triatominae speciation. Three examples are discussed. Nonetheless, sympatric speciation has also been evoked to account for the generation of particular species within cryptic species complexes. Two examples are given.

Finally, a discussion toward the benefits of relying on integrative and evolutionarily sound taxonomy approaches is offered.

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1 Toward a Unified Species Concept

The diversity of life is measured essentially in terms of number of species, even though there is an ongoing debate focusing on what a species is and how organisms speciate. The last three decades have seen prominent challenges to the current views of species concepts and species delimitation due to the advances in molecular biology and genetics (Mallet 1995). The definition of a species will depend on which species concept you choose among the 27 available definitions (Mayden 1997; Wilkins 2011). By far, the most used and widespread definition is the biological species concept (BSC), which considers species as groups of interbreeding individuals, with boundaries defined by intrinsic barriers to gene flow that have a genetic basis (Mayr 1963). The main limitation of the BSC is that populations of the same species found at a distance from each other (allopatric populations) that could not be suitably treated, because they are not in contact to randomly mate. Not even the successful crossing of allopatric populations under laboratory conditions will prove conspecificity since all ecological/geographical barriers are being removed (Claridge et al. 1985; Mallet 1995). Moreover, different cases of bona fide species hybridizing at secondary contact zones [i.e., lineages that occur at least partially in a same geographical area (sympatry) after the speciation process] are well-known. For example, although the malaria vectors *Anopheles gambiae*, *A. coluzzii*, and *A. fontenillei* (Diptera: Culicidae) are valid species, introgressed genomic regions are found that encompass genes associated with detoxification, desiccation tolerance, and olfactory perception, which are the characteristics that can alter their ability as malaria vectors (Barron et al. 2018).

Beyond the practical use of the BSC, the debate over what a species is and how it should be defined has been a matter of a long theoretical dispute among biologists. Personal expertise with respect to a particular research model or taxonomic group of interest has contributed to a "divergent radiation" in the proposal of species concepts. It is now clear that this "species definition competition" has generated more heat than knowledge. Recent countercurrent attempts have been made toward the proposal of a "unified species concept" (Table 1). Most species concepts agree in treating *existence as separately evolving metapopulation lineage* (i.e., "an inclusive population made up of connected subpopulations extended through time") as the primary defining property of the species category, but they disagree in adopting different properties acquired by lineages during the course of divergence (e.g., intrinsic reproductive isolation, diagnosability, and monophyly) as secondary defining properties (secondary species criteria). In other words, lineages do not have to be morphologically distinguishable, diagnosable, monophyletic, intrinsically reproductively isolated, ecologically divergent, or anything else to be considered species, but only

Table 1 Often-used contemporary species concepts and the properties upon which they are based (modified from de Queiroz 2007). de Queiroz's (2007) proposal is an attempt toward the unification of all species concepts presented here. Properties marked with an asterisk should be viewed as operational criteria (lines of evidence) relevant to assessing lineage separation of a single general concept that defines species as separately evolving metapopulation lineages

Species concept	Property(ies)	Advocates/References
Biological	Interbreeding (natural reproduction resulting in viable and fertile offspring)	Mayr (1942) and Dobzhansky (1950)
(reproductive) isolation	*Intrinsic reproductive isolation (absence of interbreeding between heterospecific organisms based on intrinsic properties, as opposed to extrinsic [geographic] barriers)	Mayr (1942) and Dobzhansky (1970)
Recognition	Shared specific mate recognition or fertilization system (mechanisms by which conspecific organisms, or their gametes, recognize one another for mating and fertilization)	Paterson (1985) and Masters et al. (1987)
Ecological	*Same niche or adaptive zone (all components of the environment with which conspecific organisms interact)	Van Valen (1976) and Andersson (1990)
Evolutionary	Unique evolutionary role, tendencies, and historical fate	Simpson (1951), Wiley (1978) and Mayden (1997)
Cohesion	Phenotypic cohesion (genetic or demographic exchangeability)	Grismer (1999, 2001), Templeton (1989, 1998)
Phylogenetic	Heterogeneous (see next three entries)	See next three entries
Hennigian	Ancestor becomes extinct when lineage splits	Hennig (1966), Ridley (1989) and Meier and Willmann (2000)
Monophyletic	*Monophyly (consisting of an ancestor and all of its descendants; commonly inferred from possession of shared derived character states)	Rosen (1979), Donoghue (1985) and Mishler (1985)
Diagnosable	*Diagnosability (qualitative, fixed difference)	Nelson and Platnick (1981), Cracraft (1983) and Nixon and Wheeler (1990)

to be evolving separately from other lineages (for more information, see de Queiroz 2007). It is time to put aside disagreements about species definition and focus on empirical data that can be used as evidence of lineage separation and species boundaries. Taxonomists have to agree that the definition of robust species concepts depends upon several lines of evidence, including morphological traits and ecological and molecular data.

2 Insect Diversity and Speciation

Insects are one of the most diverse group of multicellular organisms, being represented by at least 10–30 million species (Erwin 1982), which accounts for 60–65% of all living eukaryotic biodiversity (Hammond 1992). The high diversity of insect taxa is partially explained by their compact size, which allows for the occupation of small and different portions of habitats and the specialization on the use of resources that larger animals are unable to exploit (Bush and Butlin 2004). Insects are often used as model organisms in evolution research due to their relatively short generation time and the practical advantages of laboratory rearing, enabling to test speciation hypotheses with proper sample sizes (Mullen and Shaw 2014).

Speciation is a subject that has intrigued investigators for centuries. The term was coined by the American biologist Orator F. Cook in 1906, as the process by which new species arise from existing ones (Cook 1906). However, knowledge advancement on this issue has been hampered by two main limiting factors: (1) the impossibility of witnessing the phenomenon unravels in real-time (with the exception of fast-evolving viruses; Meyer et al. 2016) and (2) the difficulty in reaching a consensus regarding the understanding of what a species is and how it should be delimited.

Although alternative methods to categorize the speciation process have been proposed (cf. Butlin et al. 2008), the most used concepts rely on the geographical context of speciation, which can be assigned to three broad categories: allopatric, parapatric, and sympatric speciation methods.

Allopatric speciation occurs when an ancestral population is divided into at least two daughter populations geographically isolated; in this context, gene flow between populations is absent or, if present, largely irrelevant. Thus, these populations accumulate mutations independently, develop some degree of genetic divergence, and might become genetically isolated. A complete allopatric speciation can occur if populations of incipient species develop pre- or postzygotic barriers for reproduction. In the case of a possible secondary contact zone, selection against hybrids (reinforcement) can occur and bimodal populations (admixed local populations with a deficit of hybrid genotypes) are observed. If sexual barriers are not complete and a secondary contact zone exists between species, hybridization events occur and thus the allopatric speciation is considered incomplete, with unimodal populations (intermediate hybrid genotypes predominating).

The grasshoppers *Chorthippus brunneus* and *C. jacobsi* (Orthoptera: Acrididae) are found in Spain at a narrow band along the north coast and south of the Cantabrian Mountain, respectively. These species possibly speciated in allopatry, but have been in contact since the Pleistocenic post-glacial range expansion (Bridle et al. 2002). They can be distinguished by the number of stridulatory pegs (although there is a small degree of overlap) and different male-calling songs (Bailey et al. 2004). In the contact zone, populations with bimodal distribution are observed, with strong assortative mating, based on spatial (probably associated with habitat specialization), seasonal, and behavioral isolation (Bailey et al. 2004). Other examples on insects

illustrate hybrid zones with binomial distribution, such as observed in *Heliconius* butterflies (Lepidoptera: Nymphalidae), and ground crickets of the *Allonemobius* (Orthoptera: Gryllidae), which show strong prezygotic isolation due to assortative mating and homogamic fertilization (gamete recognition evolves faster than mate recognition), respectively (Howard et al. 1998). On the other hand, populations with unimodal distributions were observed in pine and larch budmoth host races of *Zeiraphera diniana* (Lepidoptera: Tortricidae), defined by Bush and Diehl (1982) as "populations of a species that are partially reproductively isolated from other conspecific populations as a direct consequence of adaptation to a specific host." Behavioral and molecular studies indicate that the probability of hybridization between sympatric host races is around 2–3.5% (Emelianov et al. 2003, 2004). When in sympatry, a strong genomic heterogeneity between host races in areas where hybridization occurs was observed, but no genomic heterogeneity in divergent geographical populations of the same host race. These results suggested that the divergence with gene flow is driven by selection in sympatric regions and also that low hybridization rates are sufficient to homogenize much of the genetic variation in neutral genomic regions in terms of host adaptation.

Parapatric and sympatric modes of speciation are much more controversial among molecular biologists, since considerable interspecific gene flow hampers population divergence (cf. Jiggins 2006). Because there are no clear geographical barriers, levels of assortative mating, habitat preferences, local adaptation, and hybrid fitness reduction must overcome genetic homogenization mechanisms in order to achieve speciation. Simulation models and theoretical studies proposed that high population divergence indeed requires little or no gene flow (Orr 1995; Tang and Presgraves 2009; Nosil and Flaxman 2010). In a low gene flow scenario, it is possible for populations to diverge through the fixation of adaptive mutations via positive selection (Barrett et al. 2008; Nosil and Flaxman 2010), or simply through genetic drift in small populations. In those cases, natural selection can overcome genome homogenization (through gene flow and recombination) by maintaining isolated gene pools without the intervention of geographic barriers (Turelli et al. 2001).

Parapatric speciation can be explained as an ancestral population that becomes two daughter species occupying contiguous ranges (while sympatric speciation occurs when the geographical ranges of the daughter species overlap). In both cases, speciation seems to be shaped by disruptive selection, as a consequence of favoring the evolution of specialist over generalist species through niche-partitioning or microhabitat preference. The stick insects *Timema cristinae* (Phasmatodea: Timematidae) is a great example of parapatric speciation on its course. This species inhabits southwestern North America, feeding and mating on two different host plant species that differ in foliage and general morphology. Host-specific populations have differences in morphology and can live in parapatry (Nosil 2007). Surprisingly, significantly stronger sexual isolation mechanisms seem to occur in parapatry, which means that there is a sign of ecological reinforcement (Nosil 2007). Next-generation sequencing (NGS) analysis based on thousands of Single-nucleotide polymorphism (SNPs) revealed that host adaptation leaves subtle dif-

ferentiation patterns across the genome. Moreover, divergent selection on traits not related to host use (i.e., genes not related to reproductive isolation) seems to be more relevant for generating genomic divergence between the populations. Under greater geographical separation, gradual reductions in gene flow facilitate speciation (Nosil et al. 2012).

Probably the most recognized example of sympatric speciation was observed in the apple maggot, the tephritid fruit flies sibling species complex *Rhagoletis pomonella* (Diptera: Tephritidae). Many researchers believed that the colonization of a new host in a sympatric environment and the further host preferences had started the reproductive isolation between host races based on different diapause and eclosion periods (Bush 1969; Filchak et al. 2000; Dambroski et al. 2005). From DNA sequence data of three nuclear loci and mtDNA, Feder et al. (2003) concluded that the host races became geographically isolated ~1.5 million years ago (Ma), and rare episodes of gene flow with inversion polymorphisms (restricting recombination) might have affected key diapause traits and formed adaptive clines. Therefore, these populations must have experienced a past allopatry in order to accumulate molecular changes (Xie et al. 2007) before became sympatric species. Nowadays, it is known that the barrier for gene flow remains incomplete (4–6% gene flow/generation), but most genome regions show significant geographic and host-associated variation that can account for by initial diapause intensity and eclosion time, which cause a temporal isolation between populations (Doellman et al. 2019). It is worth mentioning that sympatric populations of different host races are genetically more divergent in comparison to geographic populations within the races, which suggest that host races are being recognized as different genotypic entities in this region (Doellman et al. 2019).

The advances of molecular biology and mathematical models unveil that the geographical contextualized categories of speciation (allopatric, parapatric, and sympatric) are actually interconnected and depend on the time-frame in which they have been analyzed. As stated by Butlin et al. (2008), "At each stage of speciation, there is a spatial context on the sympatry to allopatry continuum which determines the extent of the extrinsic isolation between diverging populations." Geographical isolation reduces homogenizing gene flow and facilitates speciation events, but the evolutionary forces that shape variability are also tightly linked to the ecological factors and the mating interactions in speciation events (Fitzpatrick et al. 2009; Nosil et al. 2009).

3 Overconservative Systematics and the Paraphyly of *Triatoma*

The subfamily Triatominae is composed exclusively by hematophagous insects and seems to have evolved from predaceous Reduviidae bugs ~40 Ma (Hwang and Weirauch 2012; Ibarra-Cerdeña et al. 2014; Justi et al. 2016), which coincides with the invasion and diversification of caviomorph rodents and small marsupials (Flynn

and Wyss 1998; Poux et al. 2006; Antoine et al. 2012), and birds (Burns 1997) in South America.

This subfamily includes 150 extant and two extinct recognized species, which are classified in 16 genera and five tribes (Monteiro et al. 2018). These species occur mainly in the Americas including the Caribbean, but can also be found in southeast Australasia (Lent and Wygodzinsky 1979).

Triatoma is the most species-rich genus in the subfamily Triatominae and includes 73 species within the tribe Triatomini (Galvão and Paula 2014). Most of this diversification could be associated with cladogenetic events caused by climatic and geological changes occurred during the formation of the Americas (Hwang and Weirauch 2012; Justi et al. 2016; Monteiro et al. 2018) and can be well explained by vicariance.

Species of *Triatoma* have been clustered by several authors in different groups and complexes based on their external morphology and geographical distributions (Usinger 1944; Ryckman 1962; Usinger et al. 1966; Lent and Wygodzinsky 1979; Carcavallo et al. 2000). Since the beginning of the use of molecular markers to test evolutionary hypotheses in Triatominae, some authors have proposed rearrangements for this original classification (Schofield and Galvão 2009; de la Rúa et al. 2014; Pita et al. 2016). Some of these studies also included morphometry (de la Rúa et al. 2014) and chromosomal analysis by Fluorescence in situ hybridization (FISH) (Pita et al. 2016).

Based on new cytogenetic and morphometric data and phylogenetic results of the very important work by Hypša et al. (2002) (see below), Schofield and Galvão (2009) proposed the currently most accepted Triatomini assemblage, which subdivides species in three groups, eight complexes, and eight subcomplexes.

Historically, the use of molecular markers to study the phylogeny of *Triatoma* (Table 2) started with one or few mitochondrial genes and few representatives of these species groups and complexes (Lyman et al. 1999; García et al. 2001; Monteiro et al. 2001), advancing over time to analyze with more markers, including nuclear markers (Marcilla et al. 2001, 2002), and a growing addition of more Triatomini species (Hypša et al. 2002; de Paula et al. 2005; Hwang and Weirauch 2012; de la Rúa et al. 2014; Ibarra-Cerdeña et al. 2014; Justi et al. 2014, 2016; Pita et al. 2016). Those first studies with limited species representing the groups and complexes (Lyman et al. 1999; García et al. 2001; Monteiro et al. 2001); however, either showed weak support for the original classification based on morphological characters (Lent and Wygodzinsky 1979; Carcavallo et al. 2000) or were inconclusive (Table 2).

It was only with the analysis of a larger and taxonomically more comprehensive set of triatomine specimens that it became clearly demonstrated that the proposed species groups and complexes did not comprise reciprocally monophyletic assemblages (Hypša et al. 2002). Phylogenetic analyses based on 12S and 16S mtDNA sequencing rejected the monophyly of Triatomini rearrangements and indicated the paraphyly of *Triatoma* with respect to *Linshcosteus*, *Dipetalogaster*, *Eratyrus*, and *Panstrongylus* (Hypša et al. 2002). Table 2 shows that the number of species used in phylogenetic studies (more than the chosen markers) was decisive to establish that

Table 2 Molecular studies presenting results that can be used to reject or not the arrangements of "species groups and complexes" in Triatomini tribe. Studies were based on different markers and are showed in a progressive order of number of species (*N*) representing those Triatomini groupings plus markers (with a few exceptions)

Species groups and complexes[a]	*N*	Molecular marker	Reference
Inconclusive or weak support	9	16S, cytb	Lyman et al. (1999)
	9	16S, cytb	Monteiro et al. (2001)
	17	12S, 16S	García et al. (2001)
	12	ITS-2	Marcilla et al. (2001)
	15	ITS-2	Marcilla et al. (2002)
	10	16S, 18S, 28S, wingless	Hwang and Weirauch (2012)
Rejection[b]	43	16S	Hypša et al. (2002)
	43	16S	de Paula et al. (2005)
	18	ITS-2	de la Rúa et al. (2014)
	40	12S, 16S, COI, cytb, 18S, 28S	Ibarra-Cerdeña et al. (2014)
	27	COI, COII, cytb, 18S, 28S	Justi et al. (2014)
	52	16S	Justi et al. (2014)
	21	FISH	Pita et al. (2016)
	56	16S, 18S, 28S, wingless	Justi et al. (2016)

[a]Species groups and complexes within the genus *Triatoma* in accordance with Lent and Wygodzinsky (1979), and Schofield and Galvão (2009)
[b]Occasional support for some groups does not validate the overall arrangement

the morphological classification of groups and complexes of Triatomini was not correct ("weak support/inconclusive": 9–17 species; "rejection": 18–56 species; Table 2). In fact, it is known that the addition of molecular markers and taxa in phylogenetic analyzes should increase its accuracy (Wiens and Tiu 2012).

What followed after the important work of Hypša et al. (2002) were phylogenetic studies continuing to demonstrate the fragility of the initial morphological grouping hypotheses, but with discussions still considering at least their partial validity (de Paula et al. 2005; Hwang and Weirauch 2012; de la Rúa et al. 2014; Ibarra-Cerdeña et al. 2014; Justi et al. 2014). Further research aimed at revealing new lines of evidence to help understand relationships within Triatomini.

The addition of biogeography analyzes brought a new light to an already promising integrative taxonomic scenario (Justi et al. 2016; Monteiro et al. 2018). In a recent review, Monteiro et al. (2018) presented a new taxonomic arrangement hypothesis to represent the relationships between species groups and complexes of *Triatoma*. The hypothesis aimed to incorporate current evolutionary theories into the traditional classification scheme based on morphology (e.g., Schofield and Galvão 2009), by including new molecular, cytogenetic, morphometric, and biogeographical data published ever since (Monteiro et al. 2018).

In addition to the presentation of a rigorous and updated classification based on literature data, the authors proposed a new nomenclature consistent with the evolu-

tionary scenario that relied on two main observations: (1) studies that reinforced the paraphyly of *Triatoma* also clearly supported the existence of three lineages in Triatomini (Justi et al. 2014, 2016; Monteiro et al. 2018) and (2) the meaning of the term "species complex" in triatomine systematic studies varies depending on the context from "subgeneric assemblages defined by morphological similarity" (e.g., Lent and Wygodzinsky 1979; Schofield and Galvão 2009) to "cryptic species" (i.e., morphologically indistinguishable species; e.g., Monteiro et al. 2003).

Therefore, Monteiro et al. (2018) proposed an arrangement for the Triatomini that followed an hierarchy of: (1) three major evolutionary "lineages" composed by *Triatoma dispar*, "North American," and South American; (2) 11 "clades" within lineages defined by common ancestry and broad biogeographic correspondences; and (3) 19 "species groups" within clades, with some of these groups matching "species complexes" defined as closely related, morphologically similar or even indistinguishable species usually disclosed as a result of molecular investigations (Table 3 and Fig. 3 of Monteiro et al. 2018). The meaning of the term "species complex" in triatomine systematic studies varies depending on the context from "subgeneric assemblages defined by morphological similarity" (e.g., Lent and Wygodzinsky 1979; Schofield and Galvão 2009) to "cryptic species" (i.e., morphologically indistinguishable species; e.g., Monteiro et al. 2003).

Of the three lineages designation proposed by Monteiro et al. (2018), the "North American" lineage has the greatest morphological diversity and comprises most nominal genera (nine). In comparison, the South American lineage has only two genera: *Triatoma* and *Eratyrus*. The high morphological plasticity of Triatominae (Dujardin et al. 1999, 2009) can lead to misidentification and taxonomic uncertainties (Pita et al. 2016). However, most of the diversification seen in the "North American" lineage seems consistent with phylogenetic evidences (Galvão et al. 2003).

Although there are still many issues within Triatomini to be clarified, the accumulation of data in the literature has already shown that *Triatoma* is not monophyletic. Is it time to discuss the suitability of a taxonomic revision? Should the "North American" lineage retain the generic epithet "*Triatoma*" as it includes the type species of the genus, *Triatoma rubrofasciata*?

4 Phenotypic Plasticity and Classical Taxonomy

Phenotypic variability affects traits often used in classical taxonomy including color patterns (Abad-Franch et al. 2009; Pavan et al. 2015) or the size and shape of bodies, heads, wings (Schachter-Broide et al. 2004; Hernández et al. 2011; Nattero et al. 2013; Sandoval et al. 2015), and genital structures (Schofield and Galvão 2009). Chromatic variations of single or near-sibling species can result from adaptive plasticity, which may confound taxonomic classification. Indeed, the discovery of cryptic lineages with different vector capacity(ies) and also chromatic variants of

single species from different micro-environments were some of the greatest achievements on the taxonomy of triatomines in the early 2000s.

Rhodnius neglectus is the most abundant *Rhodnius* species in the Cerrado biome. It inhabits various palm tree species, including those of the genera *Attalea*, *Acrocomia*, *Mauritia*, *Oenocarpus*, and *Syagrus* (Gurgel-Gonçalves et al. 2004; Abad-Franch et al. 2009). This species can be misidentified as *R. nasutus*, particularly in the nearby Caatinga biome and in Caatinga-Cerrado transitional areas (Dias et al. 2008; Lima and Sarquis 2008). *Rhinacanthus nasutus* is found predominantly in the Caatinga inhabiting *Copernicia prunifera* palms (Sarquis et al. 2004), but may also be found in other palms and trees in this region (Dias et al. 2008; Lima et al. 2012). The high similarity between those two vector species and the lack of reliable diagnostic characters leads naturally to an uncertainty regarding proper taxonomic identification and determination of their geographical boundaries.

The identification of these species is based on morphological characters as chromatic patterns of body and antennae, overall body size, and male genitalia (Lent and Wygodzinsky 1979). However, Harry (1993b) detected no clear-cut differences in the male genitalia structures; in addition, important chromatic variation has been described in both species (Barrett 1995).

Abad-Franch et al. (2009) applied a geometric morphometrics aiming to differentiate *R. neglectus* from *R. nasutus* and found wing and head shape differences between these species. Some specimens from Curaçá, Bahia (Brazil) collected in *C. prunifera* palms, although phenotypically similar to *R. nasutus*, were clustered within the *R. neglectus* group, while others from the same location were clustered within *R. nasutus*. If morphometry is able to correctly assign both species, these results showed that *R. neglectus* and *R. nasutus* are sympatric in the Cerrado-Caatinga transitional area and the former species may have chromatic forms similar to those observed in the latter.

Recent observations of *Rhodnius* insects at Caatinga and Caatinga-Cerrado revealed specimens with dubious chromatic patterns. Individuals collected in *M. flexuosa* palms had a dark phenotype, a similar color to the palm fibers and base of fronds, and with coloration and diagnostic traits of *R. neglectus*. However, those collected in *C. prunifera* palms displayed a lighter chromatic pattern more similar to that of *R. nasutus* (Pavan et al. 2015). Since *R. neglectus* and *R. nasutus* may occur in sympatry (Abad-Franch et al. 2009), it raises the possibility of natural hybridization.

An alternative explanation for this observation is that *R. neglectus* would exhibit one chromatic phenotype similar to *R. nasutus* and different from the pattern described by Lent and Wygodzinsky (1979). If correct, the lighter coloration of *R. neglectus* from *C. prunifera* may be naturally selected. This coloration might have improved its chances of survival and reproduction, since they would be camouflaged with the light substrate of *C. prunifera* fibers. Therefore, populations with light phenotype increased in frequency in these palm trees, as they would be less conspicuous and thus less predated than the typical phenotypes.

Phenotypic variation was also observed for *R. nasutus*, and it seems to be governed by the microhabitat it lives in. In Ceará, Brazil, this species was collected in

five different palm tree species (Dias et al. 2008). The holotype of *R. nasutus* has a pale brownish-yellow coloring, with a red-like appearance and dark brown dots in certain regions of the body and appendices (Lent and Wygodzinsky 1979). Although populations inhabiting *C. prunifera* palms presented a reddish color, according to the original species description, other populations from *A. intumescens*, *A. speciosa*, *M. flexuosa*, and *S. oleracea* palms were chestnut-colored (Dias et al. 2008). As observed for *R. neglectus*, body coloration of *R. nasutus* specimens corresponded exactly to the fibers and base of fronds, strengthening the hypothesis that *Rhodnius* species have genes, which provide a menu of different phenotype possibilities, and the environment determines the phenotypic outcome by natural selection.

5 Tempo and Mode of Triatomine Speciation

As summarized in a recent review paper on the evolution and biogeography of the Triatominae (Monteiro et al. 2018), most studies focusing on the existence of cryptic triatomine taxa using molecular markers have often relied on two species concepts: the Biological Species Concept (BSC, Mayr 1963) and the Phylogenetic Species Concept (PSC, Cracraft 1989). We have learned that allopatric speciation seems to be the rule for most Reduviids (Monteiro et al. 2018). Here, we present three examples of triatomine speciation that probably involved vicariance and diversification with low/no gene flow among ancestral lineages. It is widely accepted that speciation is a process that requires very long time intervals to take place, usually hundreds of thousands of years (Butlin et al. 2008). With regard to the time needed for triatomines to speciate, two hypotheses were put forth that clearly challenged the *tempo* required for traditional insect speciation to occur (see below).

5.1 Fast or Slow Diversification?

Triatoma rubrofasciata and Old World Triatominae

The first hypothesis was advanced in an attempt to account for the occurrence of the six *Linshcosteus* and seven *Triatoma* species found in the Old World. It was suggested that they all descend from *T. rubrofasciata*, as a result of merchant shipping between the Americas and Asia during the sixteenth to seventeenth centuries, perhaps as a consequence of very fast (300 years) adaptive radiation processes (Schofield 1988; Gorla et al. 1997; Patterson et al. 2001; Schofield and Galvão 2009; Dujardin et al. 2015a, b). It is now well established that all Old World Triatominae are monophyletic and likely derive from a successful founding event that occurred approximately 20 Ma, with ancestral triatomine populations crossing the Bering land bridge, likely benefiting from the association with rodents, ulti-

mately reaching Eurasia (Hypša et al. (2002); Patterson and Gaunt 2010; Justi et al. 2016).

The Origin of *Rhodnius prolixus*

The second hypothesis suggested that *R. prolixus*, the most important Chagas vector in Venezuela, Colombia and parts of Central America, is a domestically adapted "derivative" of a sylvatic *R. robustus* lineage, and that speciation was the consequence of a "discrete event in Venezuela at some time after the establishment of European settlements in the 16th century" (Schofield and Dujardin 1999). This hypothesis was proposed based on morphometric (Dujardin et al. 1998, 1999) and genetic evidence (allozymes and mtDNA; Harry et al. 1992, Stothard et al. 1998, respectively), available at the time, which pointed to a lack of phenotypic and genetic variability in *R. prolixus* populations. Further research relying on better sampling of both wild and domestic *R. prolixus* populations collected from six Venezuelan states and analyzed for mtDNA and microsatellites have challenged this view by revealing high levels of genetic variation (Fitzpatrick et al. 2008).

5.2 Vicariance and Allopatric Speciation of Triatomines

Rhodnius robustus and the *Refugium* Theory

For many years, the taxonomic status of *R. robustus* was questioned due to a combination of three factors: morphological similarity, loose diagnosis, and poor sampling (cf. Monteiro et al. 2003; Pavan and Monteiro 2007). Although indistinguishable according to morphological and isozymic analyses (Harry 1993a, 1994), these species play very different epidemiological roles—*R. prolixus* is an efficient domestic vector, whereas *R. robustus* populations are entirely sylvatic. Monteiro et al. (2003) put an end to the controversy of the validity of *R. robustus* as a bona fide species through the analysis of DNA sequences of mitochondrial and nuclear markers (663-bp fragment of cytochrome b (cytb) and the D2 variable region of the 28S nuclear RNA), revealing that *R. robustus* is not only a valid species separated from *R. prolixus*, but also represents a paraphyletic complex of at least four cryptic lineages (*R. robustus* I, II, III, IV). Pavan et al. (2013) further confirmed the paraphyletic assemblage of *R. robustus* with respect to *R. prolixus* through the analysis of another nuclear marker, the fourth intron of the transmembrane protein 165 (TP165) gene. The separation of *R. prolixus* and *R. robustus* was further corroborated by a behavior study showing that nymphs of *R. prolixus* and *R. robustus* II display different locomotor activity patterns on an automated recording system (Pavan et al. 2016).

The first attempt to associate triatomine phylogeographic patterns with possible vicariant events based on molecular clock time-estimates was published in 2003 by Monteiro and collaborators. A particular and notorious example of vicariant specia-

tion is that of the *refugium* theory, advanced to account for the pattern of diversification seen in the Amazon region. The view that diversification of the Amazonian biota was caused by glaciation cycles during the Pleistocene was first introduced by Haffer (1969). The theory attempts to explain the latest of the series of differentiation events beginning in the Cenozoic that contributed to the development of the modern biota of the Amazon basin. In short, it is based on the premise that climatic changes during the Pleistocene caused rain forests to contract into isolated pockets separated by savannah. This would have confined small populations and favored their divergence by genetic drift, which would have facilitated allopatric speciation (Monteiro et al. 2003). The authors used in their phylogeographic inferences the value of 2.3% of sequence divergence per million years estimated for recently diverged arthropod taxa (Brower 1994). They concluded that all estimates between the clades within both Amazon and Orinoco regions are compatible with a Pleistocene origin and are consisted with the *refugium* theory (Monteiro et al. 2003).

Triatoma rubida and the Baja California Peninsula

Triatoma rubida was initially described as five morphologically distinguishably allopatric subspecies based mainly on chromatic differences in markings along the conexivum, distributed in Mexico and the USA: *T. rubida rubida* from the Cape region, Baja California Sur, *T. rubida cochimiensis* from Central Baja California peninsula, *T. rubida jaegeri* from Pond Island, Gulf of California, and *T. rubida sonoriana* from Sonora (all strictly Mexican subspecies); and *T. rubida uhleri* from Veracruz, Mexico, and Southwestern USA (Usinger 1944; Ryckman 1967). The "five subspecies" proposition was, however, later challenged in the 1979 revision of Lent and Wygodzinsky, who stated: "Although specimens seem to cluster around the phenotypes mentioned, not all fall easily into the categories listed above; there does seem to be a prevalence of comparatively light-colored, large-sized forms in the north and of smaller, more intensely pigmented forms in the southern part of the total range of the species. Much more abundant material than that examined by us, especially from Mexico, combined with rearing experiments, is needed for an understanding of the biosystematics of *Triatoma rubida*."

The separation of the Baja California Peninsula from mainland Mexico during the formation of the Gulf of California 5–8 Ma is believed to be the vicariant event that caused the geographic isolation of ancestral *T. rubida* populations and gave rise to *T. rubida cochimiensis* (Baja peninsula) and *T. rubida sonoriana* (Sonora). Pfeiler et al. (2006) used this geological event to calibrate the first mtDNA molecular clock for triatomines: 1.1–1.8% pairwise sequence divergence per million years (lower than the 2.3% divergence for mtDNA generally applied to insects; Brower 1994).

Triatoma dimidiata and the Isthmus of Tehuantepec

The *T. dimidiata* cryptic species complex was first recognized by Marcilla et al. (2001) based on ITS-2 sequence divergence between bugs from Yucatan and specimens from elsewhere in Mexico and from Central and South America. Following studies based on cytogenetics and genome size (Panzera et al. 2006) and mtDNA (Dorn et al. 2009; Monteiro et al. 2013) corroborated these observations. Relying on mtDNA markers (cytb and ND4), Monteiro et al. (2013) described five genetically well-differentiated, monophyletic groups (named groups I–IV plus *T. hegneri*). Their results revealed that mtDNA groups I, II, and III match, respectively, ITS-2 groups 1, 2, and 3. Group IV represented cave-dwelling Belize specimens. As pointed out by Bargues et al. (2008) and Monteiro et al. (2013), some of these genetically divergent groups clearly deserved specific status. In accordance with these orientations, the two genetically most divergent groups III and IV were recently raised to the specific level and formally described as *T. mopan* and *T. huehuetenanguensis*, respectively (Dorn et al. 2018; Lima-Cordón et al. 2019).

With regard to Groups I and II (and based on their present distribution), as the Isthmus of Tehuantepec is known to represent an important recent geological barrier for a number of sister taxa of birds, mammals, and butterflies, Monteiro et al. (2013) have suggested that the Isthmus of Tehuantepec orogeny (15–5 Ma) might have been the vicariant event responsible for the splitting of the ancestral population that led to their origin. Although groups I and II still have a subspecies status, we argue that they merit specific status.

5.3 *Parapatric/Sympatric Triatomine Speciation*

Triatoma brasiliensis Complex and the Homoploid Hybridization Hypothesis

Organisms may also speciate quite rapidly via polyploidy (Lukhtanov et al. 2015). Polyploidy (or hybrid speciation) is the term given to a set of processes whereby two species hybridize and instantly generate a third new species. They can be classified as allopolyploidy (i.e., involving a genome-doubling event that provides reproductively isolation); or homoploid hybrid speciation that occurs without an increase in ploidy (Coyne and Orr 2004).

The northeastern Brazil Chagas species complex *Triatoma brasiliensis* was comprised of three subspecies—*T. b. brasiliensis*, *T. b. macromelasoma*, and *T. b. melanica*—defined based on chromatic differences of the pronotum, legs, and hemelytra (Galvão 1956). These subspecies were, however, synonymized by Lent and Wygodzinsky (1979), who argued that intermediate forms could be found in nature. Further allozyme-based analyses showed the three subspecies were real evolutionary lineages, and yet another form was later discovered (*juazeiro* form; Costa et al. 1997). Monteiro et al. (2004) confirmed the existence of the four forms based on

mtDNA cytb phylogenetic analysis of specimens collected from the whole distribution area of the species. *Juazeiro* and *melanica* forms were raised to the specific level and formally described as *T. melanica* (Costa et al. 2006) and *T. juazeirensis* (Costa and Felix 2007). Kimura-2-parameter distances based on mtDNA evidence that bona fide sister *Triatoma* species diverge in more than 7.5% (K2P > 0.075) (cf. Monteiro et al. 2004), while intraspecific variation does not exceed 2%. Genetically less divergent sister forms *brasiliensis* and *macromelasoma* diverged in more than 2% (K2P = 0.027), and thus, were given subspecific ranks (Costa et al. 2013).

Costa et al. (2009) have analyzed morphometric, morphological, ecological, and geographic distribution data to advance the hypothesis that *T. brasiliensis macromelasoma* is a product of hybridization between the subspecies *T. b. brasiliensis* and *T. juazeirensis*. Authors acknowledge, however, that the evidence presented is not yet conclusive and that further studies are required to strengthen their claim (Costa et al. 2009). The subject has been recently revisited in a study based on chromosomal analysis, and band sizes of an ITS-1 PCR-amplified fragment (Guerra et al. 2019). Those authors also sequenced DNA from the three taxa for a fragment of the ND1 mitochondrial gene, which gave unexpectedly low pairwise genetic distances (Tamura-Nei < 0.006), pointing to a possible problem with the taxonomic identification of the specimens themselves (Guerra et al. 2019). It is well established that this magnitude of differentiation characterizes within-population or, at most, within-species levels of variation (Monteiro et al. 2004). Not surprisingly, all species showed the same cytogenetic characteristics (Guerra et al. 2019).

The speciation process of the *T. brasiliensis* complex probably involves ecological and/or temporal barriers (sympatric areas in the present may represent secondary contact zones of parapatric/allopatric populations), since they still have not evolved either pre- or post-mating barriers, as revealed by successful hybridizations in laboratory conditions (Almeida et al. 2012). New studies on ecology and genomics focusing on possible ecological selection (which would prevent backcrossing), behavioral changes (e.g., different periods of activity), or even chromosomal arrangements are still needed to clarify this issue.

The *Rhodnius pallescens—R. colombiensis*: A Case of Sympatric Speciation?

The *Rhodnius pallescens* complex is composed by three recognized species that occur in the trans-Andean region (Pacific area of South and Central America)—*R. pallescens*, *R. colombiensis,* and *R. ecuadoriensis*. *Rhodnius. ecuadoriensis* is restricted to southern Ecuador and northern Peru, occupying *Phytelephas aequatorialis* palms (Abad-Franch et al. 2009). This species is isolated from the others of this complex by geographical barriers, the Andean mountains, and probably speciated through allopatry (Galvão et al. 2003; Abad-Franch et al. 2009). *Rhodnius. pallescens* is widely distributed across Central America and Colombia in different ecological zones, inhabiting *Attalea butyracea* and *Cocos nucifera* palm

trees (Díaz et al. 2014). *Rhodnius. colombiensis* seems to be restricted to the Andean Valley of Magdalena River in central Colombia (Moreno et al. 1999). Although this species inhabits the same ecoregion and same palm tree species as *R. pallescens*, natural hybrids had not been reported (Díaz et al. 2014).

Laboratory crosses reveals the existence of both pre-zygotic and post-zygotic reproductive barriers. Female *R. pallescens* I and male *R. colombiensis* do not produce progeny, while female *R. colombiensis* and male *R. pallescens* I produce infertile F1 hybrids (Gómez-Palacio et al. 2012). Cytogenetics analyses reveal that *R. colombiensis* structural chromosomes suffered rearrangements and DNA loss in comparison to the other species of the complex (Panzera et al. 2007; Gómez-Palacio et al. 2012; Díaz et al. 2014). A clear demarcation of the biogeographical distribution of the four lineages of the complex and additional analyses within and between *R. pallescens* lineages using different molecular markers are still needed for a better knowledge on the evolutionary trends, geographical dispersion, and signs of possible adaptive radiation.

6 Toward an Integrative and Evolutionarily Sound Taxonomy

We emphasize the importance of integrating morphological, ecological, behavioral, and molecular tools to elucidate epidemiological and taxonomic unresolved questions in triatomines. Abad-Franch et al. (2013) performed an integrative taxonomic analysis to describe *Rhodnius barretti* as a new species of triatomine. They evaluated traditional morphological traits, morphometric data, and molecular phylogenetics using a fragment of cytb. This species is difficult to distinguish phenotypically from those of the *R. robustus* lineage, with the exception of the sympatric *R. robustus* II that presents chromatic (lighter coloration) and size (larger individuals) differences. However, *R. barretti* differs from *R. robustus s.l.* in the shape of both head and wings, and also in length ratios of certain anatomical structures. Moreover, phylogenetic reconstructions showed that this species is a basal member of the "*R. robustus* lineage," which encompasses *R. nasutus*, *R. neglectus*, *R. prolixus,* and the other five members of the *R. robustus* complex (Abad-Franch et al. 2013).

Besides the *R. prolixus* genome (Mesquita et al. 2015), the mitochondrial genomes of *T. dimidiata* and *T. infestans* are already available (Dotson and Beard 2001; Pita et al. 2017). As genome-sequencing is increasingly employed for non-model organisms, the ability to evaluate the taxonomic identity or status of a particular triatomine species via transcriptomes, proteomes, or metabolomes is now possible. These approaches have recently begun to be applied to triatomines, including *T. brasiliensis* (Marchant et al. 2015), *T. dimidiata* (Kato et al. 2010), *R. prolixus* (Ribeiro et al. 2014), and *T. infestans* (Traverso et al. 2016; Gonçalves et al. 2017). Genomic data were also useful for the identification of parasite, vector, and the microbiota present in *T. dimidiata* (Orantes et al. 2018). In the context of near-

sibling species and varieties of single species, Brito et al. (2019) recently synonymized *Rhodnius montenegrensis* as *R. robustus*. Most likely, the upcoming years of triatomine research will present us with the gathering of increasingly large datasets that contain separate lines of evidence from independent loci.

An alternative approach for generating genomic data at a lower cost for population genetics studies and phylogenetic analyses of closely related species is the double-digested restriction-site-associated DNA sequencing (ddRAD-seq) method. This technique was first employed as a population genomics study to infer the structuring of *R. ecuadoriensis* populations in Ecuador (Hernandez-Castro et al. 2017). This method increases the coverage of different regions of the genome and recovers reliable microsatellite and SNP data (Davey and Blaxter 2011; Kai et al. 2014).

An overlooked issue in population studies of triatomines is the difficulty of infestation foci detection, especially when colonies are small and occupy structurally complex ecotopes (Abad-Franch et al. 2010, 2014; Valença-Barbosa et al. 2014; Pavan et al. 2015). A comprehensive approach must include genetic and ecological data of triatomine species to better understand the adaptive nature of plasticity (whether is heritable and ontogenetic), and detailed frequencies of different chromatic variations in the environment (Murren et al. 2015).

New genomic tools can help explore adaptive plasticity and the following approaches deserve further attention: (1) the "omic" basis behind it, (2) comparative genomics of near-sibling species to understand its evolution, and (3) epigenetic components of inheritance that may influence plastic responses (Richards et al. 2010; Glastad et al. 2011; Zhang et al. 2013; Murren et al. 2015).

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Chromosome Structure and Evolution of Triatominae: A Review

Francisco Panzera, Sebastian Pita, and Pedro Lorite

Abstract The subfamily Triatominae, vectors of Chagas disease, represents one of the most cytogenetically studied subfamilies within the true bugs. To date, the chromosome numbers of more than 100 out of the 150 recognized species of the subfamily are known, resulting in an extremely stable group in terms of the number of autosomes (18, 20, and 22) and sex chromosome systems (mainly XY with variations in the number of X chromosomes). However, detailed analyses using laser flow cytometry, chromosome bandings, and FISH (ribosomal genes, X chromosome probes, genomic probes, and those from isolated repetitive fractions) have shown that this group is extremely diverse in their total genome content, as well as in the organization and types of sequences which constitute its chromosomes. In this chapter, we review and update current knowledge about the chromosome structure and highlight its importance to better understand the evolution of autosomes and sex chromosomes of this fascinating group of true bugs.

Keywords Chagas disease vectors · Heterochromatin · Holocentric chromosomes · In situ hybridization · Karyotype evolution · Repetitive DNAs

Abbreviations

bp	base pairs
CMA_3	chromomycin A_3
DAPI	4'-6-diamidino-2-phenylindole
FISH	Fluorescence *in situ* hybridization
GISH	Genomic *in situ* hybridization

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P.	*Panstrongylus*
pg	picograms
R.	*Rhodnius*
rDNA	ribosomal DNA
satDNA	satellite DNA
T.	*Triatoma*
TEs	Transposable elements

1 Introduction

The blood-feeding Triatominae, commonly called kissing bugs, is a subfamily of Reduviidae insects included in the infraorder Cimicomorpha, suborder Heteroptera, and order Hemiptera (Weirauch and Schuh 2011). This subfamily, distributed mainly but not exclusively in the Americas, is constituted by around 150 species of great medical relevance because most of them act as vectors of Chagas disease (Schofield and Galvão 2009; Justi and Galvão 2017; Monteiro et al. 2018).

As every hemipteran species, triatomines have holocentric chromosomes, also called holokinetic chromosomes (Schrader 1947). Holocentric chromosomes have been traditionally identified by two cytological characteristics. First, they lack a primary constriction or localized centromere, distinctive of monocentric chromosomes (Hughes-Schrader and Schrader 1961). Second, during mitotic anaphase, the sister chromatids are separated toward opposite poles with their axes parallel to each other and to the equatorial plate, a process known as holokinetic movement. The fibers of the spindle microtubules interact with most of the chromatid length, so that the chromatids do not adopt the classic V-shaped figures typically observed on monocentric chromosomes. This behavior leads to the definition of a diffuse or nonlocalized centromere (for review, see White 1973).

Holocentric chromosomes are observed in a wide variety of organisms including animals, plants, and protists (White 1973; Eichenlaub-Ritter and Ruthmann 1982; Guerra et al. 2010; Melters et al. 2012). The most well-studied holocentric species is the nematode *Caenorhabditis elegans*. In Insecta, holocentric chromosomes are present in several orders: Dermaptera, Hemiptera, Lepidoptera, Odonata, Phthiraptera, Psocoptera, Trichoptera, and Zoraptera. Other arthropods such as some spiders (order Araneae), primitive scorpions, microwhip scorpions (order Palpigradi), separate mites, and ticks also have holocentric chromosomes (Mola and Papeschi 2006). Considering the diversity of insect groups with holocentric chromosomes, several authors estimate that these chromosomes have arisen by convergent evolution at least four independent times in the evolution of Insecta (Melters et al. 2012; Drinnenberg et al. 2014; Marques and Pedrosa-Harand 2016). In order to replace a localized centromere in its regulatory role in the co-orientation of the sister chromatids, the holocentric chromosomes have adopted different strategies to ensure regular chromosome segregation in meiosis. For this reason, the meiotic

behavior and the kinetochore structures are variable among different organisms with holocentric chromosomes (Viera et al. 2009; Melters et al. 2012; Heckmann and Houben 2013; Cabral et al. 2014; Marques and Pedrosa-Harand 2016). In this chapter, we will only refer to the hemipteran chromosomes and especially to what is known in Triatominae.

In hemipteran mitosis, the spindle microtubules attach along most of the chromatid length by means of a trilaminar kinetochore (Buck 1967; Comings and Okada 1972; González-García et al. 1996a). This holocentric interaction of microtubules leads to a parallel segregation of sister chromatids during mitotic anaphase, resulting in the characteristic holokinetic movement of the holocentric chromosomes. However, hemipteran chromosomes exhibit striking differences in kinetochore structure and in their segregation during meiotic divisions. First, meiotic chromosomes do not present kinetochore structures. Multiple microtubule fibers directly interact with the chromatin of the chromosomal ends, so that anaphasic movement seems to be mediated by microtubules inserted into the chromosomes, without the participation of kinetochore plates (Buck 1967; Comings and Okada 1972). Second, the kinetic activity in meiosis is restricted to the chromosome ends in both autosomes (Hughes-Schrader and Schrader 1961) and sex chromosomes (Schrader 1935), resembling the behavior of the telokinetic chromosomes (Motzko and Ruthmann 1984). In other words, the dispersed kinetic activity in the mitotic chromosomes changes to a located kinetic activity at the chromosomal ends in the meiotic chromosomes.

Heteropteran autosomes and sex chromosomes present differences in their meiotic segregation. The first meiotic division is reductional for autosomes and equational for sex chromosomes (Ueshima 1979; Mola and Papeschi 2006; Grozeva et al. 2015). Sex chromosomes in males are achiasmatic (Solari 1979), segregating equationally during the first meiotic division and reductionally during the second one (Ueshima 1979). This post-reductional segregation, also called inverted meiosis of the sex chromosomes, is the rule in Hemiptera, although there are few exceptions (Ueshima 1979; Grozeva et al. 2006). Both in sex chromosomes and in autosomes, the kinetic activity during the first meiotic anaphase is restricted to either one of the two chromatid ends. In sex chromosomes, both chromatid ends are able to develop kinetic activity, being the election of the kinetic end a random process and independent between sister chromatids (González-García et al. 1996b; Pérez et al. 2000). However, in the autosomes, the kinetic activity of one or the other end will depend on the orientation of the bivalent during metaphase I (perpendicular or parallel to the equatorial plane). In such way the same chromosomal segment can segregate equationally or reductionally depending on the kinetically active end during the meiotic anaphases (Nokkala and Nokkala 1997; Pérez et al. 1997, 2000). Finally, the kinetic activity changes its location from one chromosome end at first meiotic division to the opposite end at the second meiotic division, irrespective of the chiasma position (Nokkala 1985; Pérez et al. 1997, 2000). Exception to this inversion in the kinetic activity was described in one autosomal pair of *Triatoma infestans*, where the same chromosomal end can be active in both meiotic divisions (Pérez

et al. 2000). An excellent review about the orientation and segregation of holocentric chromosomes in meiosis can be found in Viera et al. (2009).

2 Chromosome Numbers in Triatominae

Table 1 summarizes the triatomine species that have been cytogenetically studied so far, which include three of the five tribes (Bolboderini, Rhodniini, and Triatomini) and nine genera (of the 16 recognized). In this table, the species nomenclature follows a recently proposed classification based on phylogenetic affinities obtained by different genetic approaches (Monteiro et al. 2018). Chromosome numbers of the following species are described here for the first time: *Triatoma dispar* (Antioquia, Colombia) (Fig. 1d), *T. mopan* (Cave Rio Frio, Cayo, Belize) (Fig. 1g), *T. breyeri* (Aiquile and Mataral, Cochabamba, Bolivia) (Fig. 1k), *T. mexicana* (Guanajuato, Mexico), *T. venosa* (Santander, Colombia), and *T. recurva* (Tucson, Arizona, USA). Cytogenetic information previously reported for *Triatoma sp. affin dimidiata* from Guatemala (Petén, Ruinas Yaxhá) (Panzera et al. 2006) corresponds to the recently described *T. huehuetenangensis* (Lima-Cordón et al. 2019). Chromosomal results are presented separately for autosomes and sex chromosomes to facilitate reader interpretation.

Despite the holokinetic nature of chromosomes, which is expected to facilitate karyotype evolution through chromosomal fusions and fissions, the number of autosomes in triatomines appears to be quite stable: almost all species have a diploid autosomal number of 20. Table 1 shows that among the 102 species studied so far, 99 of them present 20 autosomes. The other three species are *T. rubrofasciata* (with 22 autosomes), *Panstrongylus megistus*, and *T. nitida* (both with 18 autosomes). Ueshima (1966, 1979) suggested that 20 autosomes are the ancestral number in Triatominae and that fission and fusion rearrangements have resulted in species with 22 and 18 autosomes, respectively. These deviations from the ancestral number are only observed in the North American lineage of the Triatomini tribe, while that in Dispar and South American *Triatoma* lineages and in the tribes Rhodniini and Bolboderini the autosomal number of 20 remains unchanged (Table 1).

In Hemiptera, the detection of structural variations such as translocations or inversions has been very rare, probably due to the lack of a morphologically differentiated centromere (Papeschi and Mola 1990; Bressa et al. 1998; Manicardi et al. 2015a). In triatomines, only two reports have described autosomal translocations that resulted in individuals with variations in the autosomal number: *Mepraia gajardoi* (Pérez et al. 2004) and *T. infestans* (Poggio et al. 2013a). In these mutant individuals, autosomal trivalent and chromosomal fragments were observed both in mitosis and meiosis.

Table 1 List of the Triatominae species cytogenetically studied until now, discriminated by diploid chromosome number (2n) in males according to the classification proposed by Monteiro et al. (2018)

Tribes and lineages	Genus: species
TRIBE BOLBODERINI	
20A + X_1X_2Y= 23	*Belminus: corredori, herreri*
TRIBE RHODNIINI	
20A + XY= 22	*Psammolestes: arthuri, coreodes, tertius*
	Rhodnius: brethesi, colombiensis, domesticus, ecuadoriensis, milesi, [*montenegrensis*], *nasutus, neglectus, neivai, pallescens, pictipes, prolixus, robustus, stali*
TRIBE TRIATOMINI	
Triatoma dispar Lineage	
20A + XY= 22	*Triatoma: boliviana, carrioni,* ***dispar, venosa***
North American Lineage	
18A + X_1X_2Y= 21	*Panstrongylus megistus; Triatoma nitida*
20A + XY= 22	*Dipetalogaster maxima;* [*Panstrongylus noireau, unpublished*]; *Paratriatoma hirsuta; Triatoma lecticularia*
20A + X_1X_2Y= 23	*Mepraia: gajardoi, parapatrica, spinolai*
	Panstrongylus: chinai, geniculatus, howardi, lignarius/herreri, rufotuberculatus, tupynambai
	Triatoma: barberi, bassolsae, confusa, dimidiata, flavida/bruneri, gerstaeckeri, hegneri, huehuetenangensis, longipennis, mazzottii, ***mexicana, mopan,*** *pallidipennis, peninsularis, phyllosoma, picturata, protracta,* ***recurva****, rubida, ryckmani, sanguisuga, sinaloensis, tibiamaculata*
20A + $X_1X_2X_3Y$= 24	*Panstrongylus: lutzi/sherlocki; Triatoma (Mepraia):* ***breyeri****, eratyrusiformis*
22A + X_1X_2Y= 25	*Triatoma: rubrofasciata*
South American Lineage	
20A + XY= 22	*Triatoma: arthurneivai, bahiensis, baratai, brasiliensis/macromelasoma, carcavalloi, circummaculata, costalimai, delpontei, garciabesi, guasayana, guazu, infestans/melanosoma, jatai, juazeirensis, jurbergi, klugi, lenti, maculata, matogrossensis, melanica, patagonica, petrocchiae, pintodiasi, platensis, pseudomaculata, rubrovaria, sherlocki, sordida, rosae* [*sordida Argentina*], [*sordida La Paz*], *vandae, williami, wygodzinskyi*
20A + X_1X_2Y= 23	*Eratyrus: cuspidatus, mucronatus*
20A + $X_1X_2X_3Y$= 24	*Triatoma: melanocephala, vitticeps*

Species analyzed here for the first time are in bold. A: Autosomes; /: synonymized species; [ ]: new proposed species

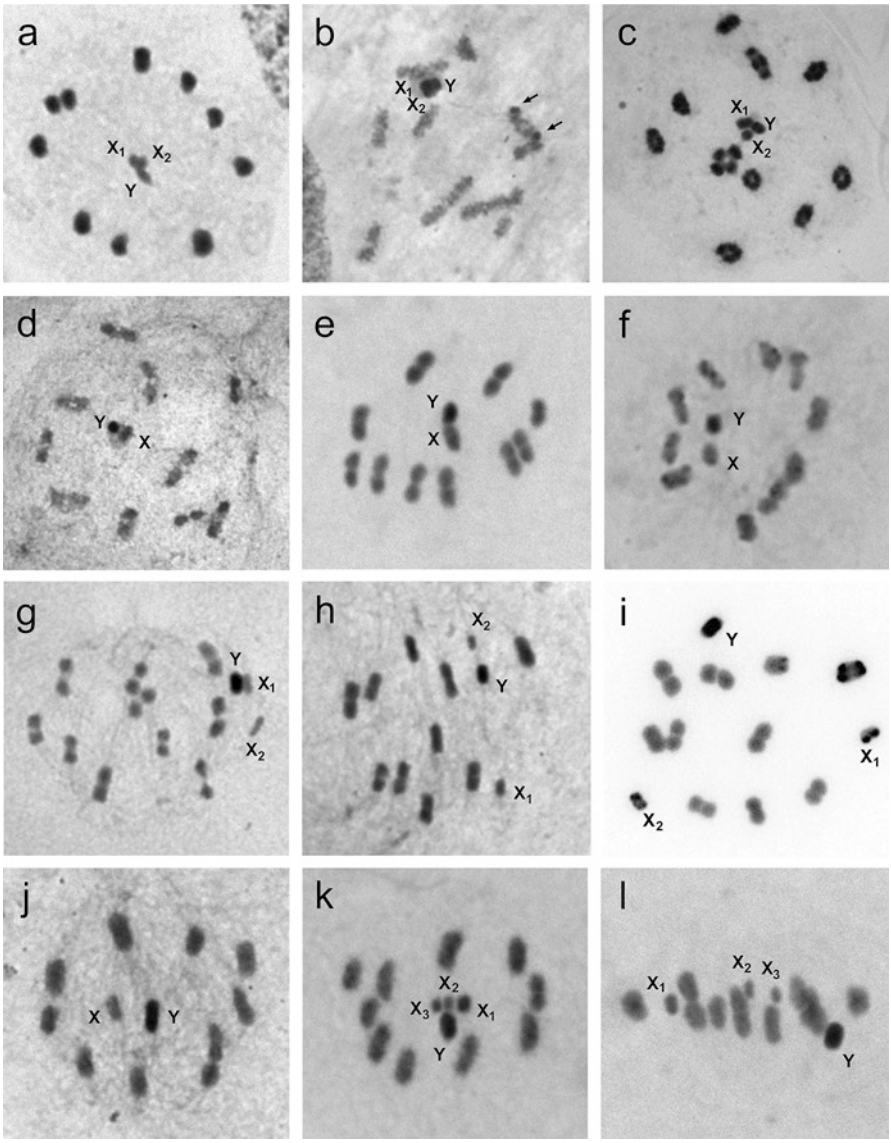

Fig. 1 Chromosome numbers in the Triatominae subfamily. C-banding. (**a**) *Belminus herreri* (2n♂= 20A + X_1X_2Y). Metaphase II. (**b**) *Eratyrus mucronatus* (2n♂= 20A + X_1X_2Y). Diplotene showing one bivalent with terminal C-blocks (arrows). (**c**) *Panstrongylus howardi* (2n♂= 20A + X_1X_2Y). Diakinesis. All autosomal pairs with conspicuous C-blocks in both chromosomal ends. (**d**) *Triatoma dispar* (2n♂= 20A + XY). Diplotene. Euchromatic X chromosome appears bigger than heterochromatic Y chromosome. Some bivalents presented small terminal C-blocks. (**e**) *T. boliviana* (2n♂= 20A + XY). Metaphase II. Similar to *T. dispar*. (**f**) *T. lecticularia* (2n♂= 20A + XY). Metaphase II. Almost all autosomes with heterochromatin. (**g**) *T. mopan* (2n♂= 20A + X_1X_2Y). Metaphase I. Y chromosome heterochromatic while both X chromosomes are euchromatic. All autosomes with small C-blocks (not observed in this stage) (**h**) *T. hegneri* (2n♂= 20A + X_1X_2Y).

3 Sex Chromosome Systems

In true bugs, the male sex is heterogametic. In triatomine species, similar to almost all heteropterans, sex chromosomes are well differentiated from autosomes by their distinct meiotic behavior: chromatin condensation throughout meiotic prophase and chromosome segregation during both meiotic divisions. In the first meiotic prophase, they are grouped together forming a positive heteropycnotic body, but they are asynaptic and achiasmatic (Solari 1979; Ueshima 1979). At metaphase I, the sex chromosomes appear clearly separated but lying side by side, without any visible physical connection between them.

Sex chromosomes present a very particular segregation in male meiotic divisions, called inverted meiosis or post-reductional segregation (Hughes-Schrader and Schrader 1961; Ueshima 1979), unlike what happens with monocentric chromosomes. In the first meiotic division, the sex chromosomes behave as univalents and the sister chromatids of each sex chromosome separate to opposite poles at anaphase I (equational division). At metaphase II, the sex chromatids belonging to different chromosomes appear associated end-to-end, forming a pseudobivalent (in XY systems) or pseudo-tri or tetravalent according to the sex system, which is located in the center of the ring formed by the autosomes (Fig. 1a, e, k). At anaphase II, these chromatids segregate to opposite poles (reductional segregation) resulting in two gametes with different sex chromosomes.

Three sex systems in males are observed in Triatominae (XY, X_1X_2Y, and $X_1X_2X_3Y$) (Fig. 1 and Table 1). Other sex mechanisms observed in others heteropterans, such as X_n0, XY_n, or neo-XY, have not been reported in triatomines. A particular sex mechanism, $X_1X_2Y_1Y_2$ has been described in *Mepraia spinolai* (Frías et al. 1998). In this case, the extra Y chromosome probably represents a chromosomal fragment or supernumerary chromosome, since it varies between the cells of the same individual, and it is also observed in females (Calleros et al. 2010; Panzera et al. 2010) (Fig. 2b).

The distribution of the sex mechanisms in Triatominae shows a correspondence with phylogenetic lineages (Table 1). All species of the tribe Rhodniini (*Rhodnius* and *Psammolestes* spp.) have an XY system, while the two Bolboderini species of the genus *Belminus* studied so far have a multiple sex system (X_1X_2Y) (Fig. 1a). In the Triatomini tribe, the three sex mechanisms are observed; although there is a strong tendency to maintain the same sex system within each lineage. The species of the *Triatoma dispar* lineage have an XY system. This is the only Triatominae

Fig. 1 (continued) Metaphase I. Similar to *T. mopan*. (**i**) *T. ryckmani* (2n♂= 20A + X_1X_2Y). Metaphase I. The three sex chromosomes, Y plus two Xs, with heterochromatin. Furthermore one or two autosomal pairs with terminal C-blocks. (**j**) *T. williami* (2n♂= 20A + XY). In the chromosome complement, only the Y chromosome is heterochromatic. (**k**) *T. breyeri* (2n♂= 20A + $X_1X_2X_3Y$). Metaphase II. All autosomes with small C-blocks (not observed in this stage). The Y chromosome is heterochromatic and the three X chromosomes with intermediate staining. (**l**) *T. eratyrusiformis* (2n♂= 20A + $X_1X_2X_3Y$). Metaphase I. Similar to *T. breyeri*

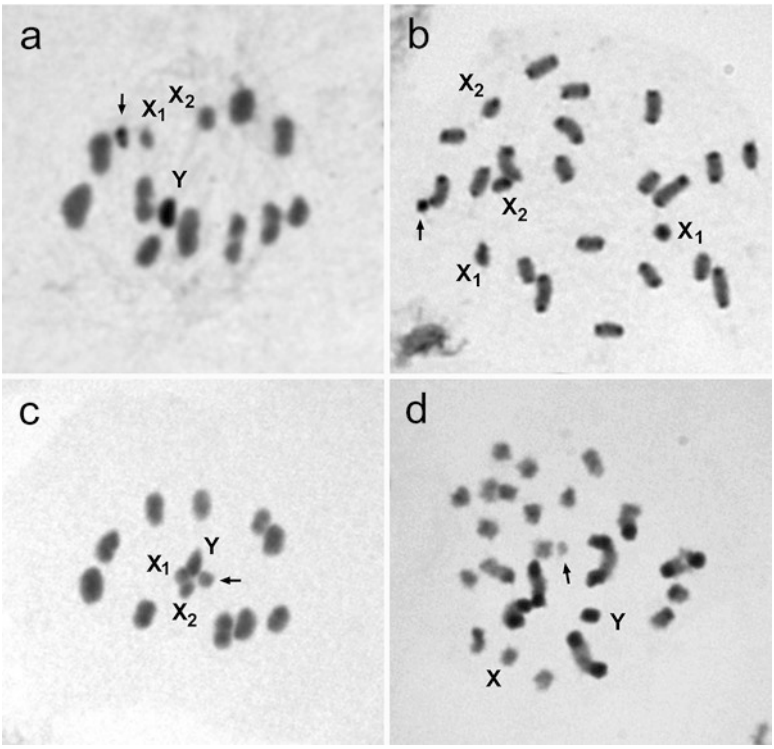

Fig. 2 Supernumerary or B chromosomes (arrows) in Triatominae subfamily. (**a**) *Triatoma longipennis* (2n♂= 20A + X_1 X_2Y). Metaphase I. C-banding. Heterochromatic B chromosome. (**b**) *Mepraia spinolai* (2n♀= 20A + $X_1X_1X_2X_2$). Oogonial mitosis. C-banding. Heterochromatic B chromosome. All autosomes and X chromosomes with C-heterochromatic blocks. (**c**) *M. spinolai* (2n♂= 20A + X_1X_2Y). Metaphase II. Giemsa staining. B chromosome close to sex chromosomes. (**d**) *T. infestans* (intermediate chromosomal group with four autosomal pairs plus Y chromosome with C-heterochromatin) (2n♂ = 20A + XY). Spermatogonial mitosis. C-banding. Euchromatic B chromosome

group where the X chromosome is a bit larger than the heterochromatic Y chromosome (Figs. 1e and 3a). All species of the South American lineage also have the XY system, except for four species with multiple X chromosomes: *Eratyrus cuspidatus* and *E. mucronatus* (with X_1X_2Y, Fig. 1b) and *T. melanocephala* and *T. vitticeps* (with $X_1X_2X_3$Y, Fig. 3b). On the contrary, all species of the North American lineage present multiple systems (X_1X_2Y and $X_1X_2X_3$Y) except four species with XY system (*Dipetalogaster maxima, Panstrongylus noireau, Paratriatoma hirsuta*, and *T. lecticularia*) (Fig. 1f).

Although XY mechanism is the most frequent in Hemiptera, the question of whether the X0 (Ueshima 1979) or XY (Nokkala and Nokkala 1983) mechanism is the ancestral in this group is still unresolved. However, it is accepted that the multiple sex chromosome systems in Hemiptera have evolved from the XY system

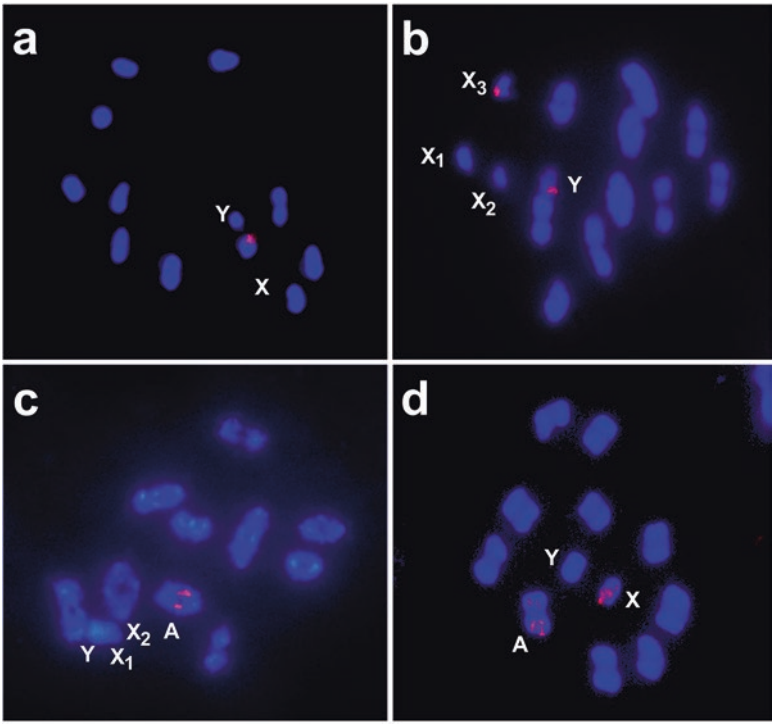

Fig. 3 Four location patterns of 45S ribosomal DNA clusters described in Triatomine subfamily using 18S rDNA probe by fluorescence *in situ* hybridization (FISH). (**a**) Pattern X chromosome. *Triatoma boliviana* (2n♂= 20A + XY). Metaphase II. The X chromosome appears clearly larger than the Y chromosome, a distinctive feature of the species of the dispar lineage. (**b**) Pattern X plus Y chromosome. *T. vitticeps* (2n♂= 20A + $X_1X_2X_3Y$). Metaphase I. (**c**) Pattern one autosomal pair. *Panstrongylus lignarius/herreri* (2n♂= 20A + X_1X_2Y). Diakinesis. Terminal heterochromatic blocks (DAPI+) are observed in almost all bivalents. (**d**) Pattern one autosomal pair plus X chromosome. *T. delpontei* (2n♂= 20A + XY). Metaphase I

(Ueshima 1979). For this reason, many authors have proposed for Triatominae as well as for Heteroptera that the multiple X chromosome systems have arisen by fragmentation (fission) of the original X chromosome (Payne 1909; Ueshima 1966; Panzera et al. 2010; Alevi et al. 2017). Other mechanisms such as non-disjunction of X chromosomes (assumed for *Cimex*, Ueshima 1979) or chromosomal fragments (resulting by an autosomal translocation, Pérez et al. 2004) have also been suggested.

The detection of a stable polymorphism in the number of sex chromosomes in a reduviine *Zelurus femoralis*, close to Triatominae, strongly supports that the fragmentation is the most likely origin of multiple X chromosomes in Reduviidae. In this species, Poggio et al. (2013b) detected the coexistence, within the same population, of males with two sex systems: XY and X_1X_2Y. Considering that the new variant (X_1X_2Y) exhibits normal segregation during meiosis and that it is present in high frequency of individuals analyzed for several years, these authors suggested that

this new sexual system is stable and its origin is not recent. Poggio et al. (2013b) also concluded that this polymorphic population of *Z. femoralis* represents a direct evidence of the emergence of multiple sex chromosomes through the fragmentation of a single X chromosome.

Considering the variability in size, staining, and sequence composition of the X chromosomes (Bardella et al. 2016a; Panzera et al. 2010; Pita et al. 2017a, 2018, see Sect. 7.6.), as well as the occurrence of the same multiple sex system in phylogenetically distant species, it is very likely that the fission processes of the original X chromosome have occurred several times during the evolution of triatomines. An illustrative example is the presence of the $X_1X_2X_3Y$ system in five not closely related triatomine species, included in North and South American lineages: *T. breyeri* (Fig. 1k), *T. eratyrusiformis* (Fig. 1l), *T. melanocephala*, *T. vitticeps* (Fig. 3b), and *Panstrongylus lutzi* (Table 1). Molecular phylogenies (García et al. 2001; Hypša et al. 2002; Campos et al. 2013; Justi et al. 2014) suggest that the first two species form a monophyletic clade with *Mepraia spp*., which have X_1X_2Y system. Probably the three X chromosomes of *T. breyeri* and *T. eratyrusiformis* are derived from a fission process that occurred in a common ancestor with the *Mepraia* species. In South American *Triatoma*, the close relationship between *T. melanocephala* and *T. vitticeps* also suggests that they are derived from a common ancestor (Justi et al. 2016), but one different from *Mepraia* species. Finally, *Panstrongylus lutzi* is evolutionarily distant from the remaining species with $X_1X_2X_3Y$ mechanism. In conclusion, the formation of three X chromosomes has probably arisen independently at least three different times during Triatominae evolution. This conclusion is also supported by the variability observed in the chromosomal location of the ribosomal genes in species with the $X_1X_2X_3Y$ sex system, that show three different patterns among the five studied species (Panzera et al. 2012 and unpublished data) (Table 2).

On the other hand, the fusion between multiple X chromosomes originating an XY system also seems to have occurred several times in Triatominae. Almost all species of the North American lineage present multiples X chromosomes (X_1X_2Y and $X_1X_2X_3Y$ systems), being X_1X_2Y considered as the ancestral state for this group (Panzera et al. 2010). Within this lineage only three species have a XY system: *Dipetalogaster maxima*, *Triatoma lecticularia* (Fig. 1f), and *Paratriatoma hirsuta* (Ueshima 1966). Despite belonging to different genera, these three species constitute a monophyletic group, clearly differentiated from other species of this lineage (Hypša et al. 2002; Justi et al. 2014, 2016). It is very likely that the XY system of these three species is a derived character originated by a fusion of the X_1 and X_2 chromosomes in the common ancestor. A similar but independent process could have also happened in a new described species of *Panstrongylus*, provisionally called *P. noireau*, sister of *P. rufotuberculatus* (unpublished). This species has a simple sex system XY, instead of multiple X chromosomes present in all other *Panstrongylus* species (see Table 1). In conclusion, the fusion of the ancestral X_1 and X_2 chromosomes to originate an XY system has taken place at least two times in the North American lineage: once in the clade comprised by the three previously mentioned species and other in *P. noireau*.

Table 2 Chromosomal location of the major ribosomal DNA clusters in triatomine species studied until now, discriminated by diploid chromosome number (2n) in males according to the classification proposed by Monteiro et al. (2018)

Tribes and lineages Diploid number (2n)	Chromosome Location of rDNA clusters	Genus: species
TRIBE RHODNIINI		
20A + XY = 22	X chromosome	*Rhodnius: colombiensis, nasutus, prolixus, robustus*
	X and Y	*Psammolestes: tertius*
		Rhodnius: domesticus, milesi, neglectus, neivai, pallescens, pictipes, stali
	Polymorphic (X alone, X and Y)	*Rhodnius ecuadoriensis*
TRIBE TRIATOMINI		
Triatoma dispar Lineage		
20A + XY = 22	X chromosome	*Triatoma: boliviana, carrioni*
North American Lineage		
18A + X_1X_2Y= 21	One autosomal pair	*Panstrongylus megistus, Triatoma nitida*
20A + XY= 22	One autosomal pair	*Triatoma lecticularia*
20A + X_1X_2Y= 23	One autosomal pair	*Panstrongylus: chinai, lignarius/herreri, howardi*
		Triatoma: dimidiata, flavida/bruneri, huehuetenangensis, mazzottii, pallidipennis, phyllosoma, protracta, tibiamaculata
20A + $X_1X_2X_3Y$= 24	One autosomal pair	*Panstrongylus lutzi/sherlocki;* (unpublished)
		Triatoma: breyeri (unpublished), *eratyrusiformis* (unpublished)
22A + X_1X_2Y= 25	One autosomal pair	*Triatoma rubrofasciata*
20A + XY= 22	X chromosome	*Dipetalogaster maxima*
20A + X_1X_2Y= 23	X_1 chromosome	*Mepraia: gajardoi, spinolai*
20A + XY= 22	X and Y	[*Panstrongylus noireau*](unpublished)
South American Lineage		
20A + XY= 22	One autosomal pair	*Triatoma: baratai, brasiliensis/macromelasoma, carcavalloi, circummaculata, costalimai, guasayana, guazu, jatai, klugi, patagonica, pintodiasi, pseudomaculata, rubrovaria, sherlocki,* [*sordida La Paz*], *williami*, *wygodzinskyi*
20A + XY= 22	X chromosome	*Triatoma: garciabesi, platensis, sordida*
20A + XY= 22	X and Y	*Triatoma: jurbergi, maculata, matogrossensis, rosae* [*sordida Argentina*], *vandae*
20A + XY= 22	X and one autosomal pair	*Triatoma delpontei*

(continued)

Table 2 (continued)

Tribes and lineages Diploid number (2n)	Chromosome Location of rDNA clusters	Genus: species
20A + XY= 22	Polymorphic	*Triatoma infestans*
20A + X_1X_2Y= 23	X_1 and Y	*Eratyrus cuspidatus*
20A + $X_1X_2X_3Y$=24	X_3 and Y	*Triatoma vitticeps*
20A + $X_1X_2X_3Y$=24	X_1	*Triatoma melanocephala* (unpublished)

Data extracted from Severi-Aguiar and Azeredo-Oliveira (2005), Severi-Aguiar et al. (2006), Morielle-Souza and Azeredo-Oliveira (2007), Bardella et al. (2010), Panzera et al. (2012, 2014, 2015), Pita et al. (2013, 2016a, b), Dujardin et al. (2015), Hieu et al. (2019), Villacís et al. (2020)
A: Autosomes; /: synonymized species; [ ]: new proposed species

Several authors have used the terms agmatoploidy (Malheiros-Gardé and Gardé 1950) and symploidy (Luceño and Guerra 1996) to refer to the fusion or fission between the few chromosomes that occur in Heteroptera (Bardella et al. 2012; Gallo et al. 2017; Alevi et al. 2018a). This terminology, originally defined for the holocentric chromosomes of plants (*Luzula*, Juncaceae), involves the fragmentation and fusion of an entire chromosome complement, generating a diploid with twice or half the chromosomes of the original species. A recent review shows that cases of doubling the number of chromosomes by agmatoploidy are exclusively limited to plant species and its occurrence in animals has never been proven (Guerra 2016). For Heteroptera, Thomas (1987) suggested that there is no evidence that agmatoploidy acts as mechanism of speciation in this insect group. Both processes do not occur in triatomines with an almost unchanged chromosome number, so these terms are not valid for this insect group. The concepts of partial agmatoploidy and partial symploidy are applied erroneously for processes where few chromosomes participate (Alevi et al. 2017, 2018a), being indistinguishable from chromosomal fissions and fusions. For these reasons, Guerra (2016) proposed that the terms agmatoploidy and symploidy should be avoided or even abolished in animal species.

4 B Chromosomes

Another source of intraspecific chromosome number variation is the presence of B chromosomes, also known as supernumerary, accessory, or extra chromosomes. These particular chromosomes are dispensable elements reported in about 15% of eukaryotic organisms. They do not recombine with chromosomes of the standard complement and follow their own pathway of transmission (Camacho 2005). In addition, they are frequently heterochromatic, generally segregate irregularly, and are often present in varying numbers, both between individuals of the same or

different populations and even among cells of the same individual. In true bugs, B chromosomes have been reported in a dozen species (Kuznetsova et al. 2011).

Within Triatominae, B chromosomes have been detected in three species: *T. longipennis, M. spinolai,* and *T. infestans* (Panzera et al. 2010). In *T. longipennis,* these were detected in a few male individuals having a single heterochromatic B chromosome present in both mitotic and meiotic cells. In the first meiotic division, the additional chromosome behaved as univalent and did not alter the anaphasic segregation of the autosomes or sex chromosomes. In metaphase II, a B chromosome was positioned close to the sex chromosomes (Fig. 2a). In males and females of *M. spinolai,* both in mitosis and meiosis, one to three supernumerary chromosomes (euchromatic and/or heterochromatic) were observed, with a very variable frequency even within the same individual (Fig. 2b). The B chromosomes of *M. spinolai,* unlike in *T. longipennis,* could affect the normal segregation of the autosomes and sex chromosomes during both meiotic divisions. Thus, metaphase II plates appear with a variable number of autosomes and/or sex chromosomes, producing non-viable gametes and affecting the fecundity of the carrier individual (Fig. 2c).

In pyrethroid-resistant populations of *T. infestans* from Argentina (Salta, Salvador Mazza) and Bolivia (Tarija), a high frequency of individuals (males and females) were reported to bear chromosomal fragments, possibly B chromosomes (Panzera et al. 2014). These chromosomes, which could be euchromatic or heterochromatic, were observed only in gonial mitosis (Fig. 2d) but not in meiosis. Male individuals carrying B chromosomes exhibited a normal meiotic behavior. In the aphid *Myzus persicae*, an autosomal translocation is responsible for an organophosphate and carbamate resistance by the amplification of esterase gene, due to a position effect caused by the repositioning of the heterochromatin (Blackman et al. 1978). In *T. infestans,* those B chromosomes are possibly the consequence of autosomal translocations that may modify the gene expression of euchromatic regions adjacent to the heterochromatin (position-effect variegation) (Panzera et al. 2014).

These few examples exemplify the extensive variability of B chromosomes in Triatominae in terms of their number, size, staining, frequency, meiotic behavior and particularly, in their genetic consequences. This variability also suggests that the B chromosomes in Triatominae have originated by different mechanisms. A better characterization of repetitive DNA sequences and single-copy genes located on B chromosomes of triatomines could clarify the origin and evolution of these particular chromosomes, as it has been carried out in other insects (Ruiz-Estevez et al. 2012; Bueno et al. 2013; Manrique-Poyato et al. 2015; Silva et al. 2021).

5 Genome Size in Triatomines

The amount of haploid genomic DNA (C-value) in Triatominae was initially determined by densitometry techniques (Schreiber et al. 1972; Panzera et al. 1995) and later by laser flow cytometry (Panzera et al. 2004, 2007; Gómez-Palacio et al. 2012; Díaz et al. 2014). The analysis of 21 species from five genera showed that the

haploid genome size in Triatominae varies up to fourfold, from around 0.72 pg (in three *Rhodnius* species) to 2.90 pg (*T. delpontei*). The average genomic DNA content in Triatomini species was 1.25 pg, equivalent to 1.22×10^9 bp (Panzera et al. 2007, 2010). Particularly interesting is the intraspecific variation observed in *T. infestans* populations, where there are substantial differences (from 30% to 50%) in the amount of nuclear DNA (Panzera et al. 2004; Bargues et al. 2006). Given that the analyzed species present similar male diploid chromosome number (22 or 23 chromosomes), the broad range in the total DNA contents is probably due to the smaller size of the *Rhodnius* chromosomes and different amounts of heterochromatin within Triatomini tribe species.

6 Cytogenetic Studies of Hybrids

There is an extensive bibliography on natural and experimental hybrids in triatomines (for references, see Campos-Soto et al. 2016). Experimental hybridization represents a powerful tool for demonstrating the existence of several isolating mechanisms that limit gene flow between animal species. Although natural hybridization usually results in non-viable offspring, its importance in speciation as an adaptation mechanism in the formation of new species has been recognized (Arnold 1997). In triatomines, it has been suggested that some species of the *Triatoma brasiliensis* complex have arisen by a special type of hybridization called homoploid hybrid speciation (Coyne and Orr 2004), that is, hybridization without a change in chromosome number (Costa et al. 2009; Correia et al. 2013; Lukhtanov et al. 2015; Guerra et al. 2019). This mode of speciation probably also has happened in other triatomine groups that present a wide reproductive compatibility, such as in some species of the phyllosoma complex (Martínez-Ibarra et al. 2008, 2009).

Recently, experimental hybridization in triatomines was used to establish the taxonomic status among putative sibling species or differentiated populations (Martínez-Hernandez et al. 2010; García et al. 2013; Mendonça et al. 2014, 2016; Campos-Soto et al. 2016; Nattero et al. 2016; Alevi et al. 2018b; Nascimento et al. 2019). Adult progeny resulting from interspecific crossings is a frequent phenomenon in triatomines and has been observed in several species of the genera *Mepraia*, *Rhodnius, Triatoma*, and recently in *Panstrongylus* (Villacís et al. 2020). Unlike what is reported in mammals and insects such as *Drosophila* (Yamamoto 1993), all interspecific crossings in triatomines yielded adult hybrids of both sexes, not following Haldane's rule (Haldane 1922). The vast majority of these hybrids are sterile (unable to produce viable offspring), while others may be totally fertile or have different degrees of infertility.

Cytogenetic analyses of adult F1 hybrids began in the 1950s with the pioneer studies of Giorgio Schreiber in Minas Gerais, Brazil (Schreiber and Pellegrino 1950). The main objective of the chromosome analysis of the F1 hybrids was to establish whether these adults were capable of producing balanced gametes, a necessary requirement but not enough to generate offspring. The meiotic analysis of the

hybrids allows to determine the degree of genetic homology among the chromosomes of the parental species by observing the chromosomal pairing between the homeologous chromosomes.

Chromosome analysis of interspecific hybrids has included the following crosses: *Triatoma infestans*/ *T. rubrovaria* in both directions (Schreiber and Pellegrino 1950; Scvortzoff et al. 1995; Pérez et al. 2005), *T. infestans* ♂/ *T. pseudomaculata* ♀ (Schreiber et al. 1974), *T. sordida*/ *T. pseudomaculata* in both directions (Schreiber et al. 1975), *T. infestans*/ *T. platensis* in both directions (Scvortzoff et al. 1995; Pérez et al. 2005), *T. platensis* ♂/ *T. delpontei* ♀ (Pérez et al. 2005), *T. protracta* ♂/ *T. barberi* ♀ (Ueshima 1966), *T. sherlocki*/ *T. lenti* in both directions (Mendonça et al. 2014*), T. lenti* ♂/ *T. bahiensis* ♀ (Alevi et al. 2018b), *M. spinolai* ♂/ *M. gajardoi* ♀ (Campos-Soto et al. 2016), and *Rhodnius pallescens* ♂/ *R. colombiensis* ♀ (Díaz et al. 2014). Except for the crossings between *T. infestans*/ *T. platensis*, *T. platensis*/ *T. delpontei*, *T. sherlocki*/ *T. lenti,* and *M. spinolai* ♂/ *M. gajardoi* ♀, all the remaining F1 hybrids presented different degrees of abnormalities in the meiotic pairing, recombination failures, and irregular chromosome segregations, resulting in genetically unbalanced gametes and consequently the individuals were almost or completely sterile.

Triatoma infestans, *T. platensis,* and *T. delpontei* share a recent common ancestor, constituting the infestans clade (Monteiro et al. 2018), previously called infestans subcomplex (Schofield and Galvão 2009). The viability of hybrids between *T. infestans* and *T. platensis* has been maintained for several generations, including backcrosses, which suggests that these species have not developed morphological or genetic barriers to avoid exchange (Usinger et al. 1966; Scvortzoff et al. 1995; Pérez et al. 2005). Furthermore, the low genetic distances between them (Pereira et al. 1996; Bargues et al. 2006) and the occurrence of natural hybrids indicate that introgression events are likely (Abalos 1948; Usinger et al. 1966; Franca 1985). In the crosses between *T. platensis* and *T. delpontei,* the situation is very different since partial sterility is observed between both species. Adult progeny is only obtained when *T. delpontei* was the female progenitor, revealing a pre-zygotic isolation related to mating (Pérez et al. 2005). In the crosses between *T. delpontei* and *T. infestans* we obtained viable F1 hybrids only when *T. delpontei* was the male progenitor (unpublished). This successful crossing is exactly the opposite to what is observed between *T. platensis* and *T. delpontei* (only viable when *T. delpontei* was the female). These F1 hybrids (males and females) were fertile with each other and with some of the backcrosses with the parental species. Meiotic analyses of F1 males showed a very variable frequency of chromosomal abnormalities among individuals. Some of them produce normal gametes while others show a high percentage of genetically unbalanced cells due to irregularities in chromosomal pairing.

The crosses between *T. sherlocki/T. lenti* yielded adult F1 hybrids, with completely normal meiosis (Mendonça et al. 2014). However, the F2 adults have irregularities in their meiosis, with severe anomalies in the chromosome pairing. Therefore, F2 individuals are almost completely sterile, unable to produce F3 adults (Mendonça et al. 2014). This abnormal chromosomal behavior in F2 individuals may be one of the causes that result in the so-called hybrid breakdown. This process is a

post-mating isolating mechanism defined as inviability or sterility in the F2 or later generations of interspecific crosses, even though the F1 hybrids are viable and fully fertile (Johnson 2010). A similar situation was observed in the crossing between *Mepraia spinolai/M. gajardoi* because in this case hybrid breakdowns are observed in the second backcrosses, in addition to other hybrid incompatibilities (Campos-Soto et al. 2016). In conclusion, the cytogenetic analysis of adult hybrids allows to determine if there is chromosomal incompatibility between the parental species. However, the detection of F1 hybrids with normal and fertile gametes does not guarantee that subsequent generations are viable and fertile.

7 Longitudinal Differentiation of Triatomine Chromosomes

In spite of the extensive stability in their autosomal number, triatomines exhibit a great variability of the genome size, chromosome location of ribosomal clusters, and in the amount, distribution, and composition of the repetitive sequences, revealed by C- and fluorescence bandings and DNA probes (see below). However, unlike what happens in other hemipteran families, the analysis of others of repetitive chromosomal markers, such as 5S rDNA, U1, and U2 snDNA or histone genes, failed to obtain satisfactory results in this insect group (Mandrioli and Manicardi 2013; Manicardi et al. 2015b; Anjos et al. 2016, 2018, 2019; Bardella et al. 2016b; Bardella and Cabral-de-Mello 2018).

7.1 C-Banding

The first report that detected longitudinal variation in the triatomine chromosomes was performed in *T. infestans* (Panzera et al. 1992) by the application of C-banding technique that revealed constitutive heterochromatin (C-heterochromatin) (Sumner 1972). This particular class of chromatin is mainly composed of repeated DNA, both organized in tandem (satellite DNA) and dispersed (transposable elements), being a fundamental component in the structure and functionality of chromosomes (Charlesworth et al. 1994). Changes in the amount and location of heterochromatin generally alter chromosomal recombination and segregation, and even the expression of genes included in both the heterochromatin and adjacent euchromatic regions (position effect variegation) (Verma 1988; Dimitri et al. 2009).

The distribution of C-heterochromatin has been analyzed in around a hundred triatomine species, showing that this cytogenetic marker is very useful to characterize and differentiate triatomine species (Pérez et al. 1992, 2004; Panzera et al. 1995, 1997, 1998, 2004, 2006, 2015; Crossa et al. 2002; Dujardin et al. 2002, dos Santos et al. 2007; Calleros et al. 2010; Alevi et al. 2013, 2014, 2015; Nattero et al. 2016; Pita et al. 2016a). All species showed a Y chromosome almost totally heterochromatic, and in some species the X chromosome has also heterochromatic blocks

(Fig. 1) (Panzera et al. 2010). Autosomal C-heterochromatin was described in almost all studied genera (*Eratyrus, Mepraia, Panstrongylus, Rhodnius,* and *Triatoma*), including striking interspecific variations in the total amount (can represent up to 45% of the autosomal complement), size of C-blocks (in *T. nitida* the C-block represents over 80% of the whole chromosome), number of chromosomes carrying heterochromatin (from none to all autosomes), and behavior of C-blocks during both mitotic and meiotic divisions (Panzera et al. 2010). In general, they have a terminal location (in one or both chromosomal ends) but intercalary positions have also been reported (Panzera et al. 1992, 1995, 1997).

At the evolutionary level, species complexes, as assigned by Monteiro et al. (2018), tend to have the same heterochromatic pattern (similar amount and distribution of C-blocks). In the North American Triatomini lineage, two clearly differentiated groups are observed: a) autosomes without C-heterochromatin (or very small C-blocks) including all species of dimidiata, phyllosoma, and flavida/*Nesotriatoma* species complexes; and b) autosomes with C-blocks in both chromosomal ends of all autosomal pairs: *T. barberi*, *T. lecticularia*, *T. protracta*, *T. sanguisuga,* and *T. rubrofasciata*. In the *Panstrongylus* genus two chromosomal trends are recognized (Crossa et al. 2002; unpublished data): with autosomal heterochromatin (*P. chinai*, *P. howardi*, *P. herreri/lignarius, P. rufotuberculatus, P. noireau*) and without heterochromatin (*P. megistus*, *P. lutzi/sherlocki*, *P. tupynambai*), and one polymorphic species depending on its geographic origin (*P. geniculatus*). A similar differentiation also occurs in the South American Triatomini lineage: species with little or without heterochromatin (rubrovaria and pseudomaculata complexes, and *Eratyrus* genus), and species with large amounts of heterochromatin (brasiliensis and infestans complexes). Species groupings according to their heterochromatic patterns agree with the evolutionary trends determined by nuclear and mitochondrial DNA sequences (Monteiro et al. 2018).

On the other hand, intraspecific variation or polymorphisms in the amount of C-heterochromatin was reported in several species: *T. infestans* (Panzera et al. 1992, 2004, 2014), *T. dimidiata* (Panzera et al. 2006), *T. sordida* (Panzera et al. 1997), *T. patagonica* (Nattero et al. 2016), *P. geniculatus* (Crossa et al. 2002), and *R. pallescens* (Gómez-Palacio et al. 2008, 2016). Some of these variations were analyzed with other genetic markers (isoenzymes and DNA sequences) in such a way that the heterochromatin variants turned out to be cryptic species, as in *T. dimidiata* (Bargues et al. 2008; Monteiro et al. 2013) and *T. sordida* (Jurberg et al. 1998; Noireau et al. 1998; Panzera et al. 2015). In *T. patagonica* and *P. geniculatus* more detailed analyses are still pending to confirm whether the heterochromatin variants are really different populations or sibling species.

The most striking intraspecific variation of C-heterochromatin was reported in *T. infestans*, main vector of Chagas disease in South America. Extensive population studies from Argentina, Brazil, Bolivia, Chile, Paraguay, Peru, and Uruguay revealed two main chromosomal groups. These groupings were designated as "Andean" and "non-Andean" due to their geographic occurrence, with a hybrid zone between them (intermediate group) (Panzera et al. 1992, 2004, 2014). The "Andean"' group shows 50% more heterochromatin than the "non-Andean" group,

which is correlated with differences in the total genome content (Panzera et al. 2004). Based on this striking variation of C-heterochromatin and chromosomal location of ribosomal clusters (see Sect. 7.3.), we have postulated an Andean origin of *T. infestans* (Panzera et al. 2004, 2014). In contrast, recent analyses with nuclear and mitochondrial sequences suggest a Chaco origin (Fernández et al. 2019), although these markers are not conclusive about resolving the origin and spread of *T. infestans* (for review see Torres-Pérez et al. 2011).

7.2 Fluorochrome Banding

The application of C-banding and subsequent fluorochrome staining with DAPI and CMA_3 allows a better characterization of heterochromatic regions in terms of their relative enrichment with AT or GC base pairs, respectively. The fluorescent C-banding analysis of 41 species from six different genera allowed a clear differentiation of the heterochromatic regions classified as similar by C-banding, both in autosomes and sex chromosomes (Pérez et al. 1997, 2000; Severi-Aguiar et al. 2006; Bardella et al. 2010, 2014a, 2016a). We described five fluorescent C-banding patterns in autosomes, five in X chromosomes, and two in the Y chromosome (Bardella et al. 2016a). Heterochromatic autosomal regions can consist of sequences exclusively stained with DAPI, exclusively CMA_3+, co-localized (positive for DAPI and CMA_3 simultaneously), or can be subdivided into two sub-regions: one DAPI+ and another CMA_3+. Some species presented autosomes with more than one of the patterns described above. Although most species bear euchromatic X chromosomes, some species might present DAPI+, CMA_3+, or both fluorochrome-enriched blocks. In species with multiple Xs, different patterns of fluorochrome staining can be observed between the X chromosomes. All species of Triatomini tribe showed a heterochromatic Y chromosome entirely DAPI+, while in Rhodniini species the Y chromosome did not display fluorescent signals. In some Triatomini species, the DAPI+ Y chromosome also included a CMA_3+ region, associated with ribosomal clusters.

The diversity of fluorochrome profiles is probably due to the amplification of different families of repeated sequences, reflecting an extraordinary dynamic of change in the Triatominae genomes. This variability contrasts with that observed in other heteropteran groups, in which each subfamily presents few fluorochrome patterns (Bressa et al. 2005; Bardella et al. 2012, 2014b, c, d).

7.3 Chromosomal Location of Ribosomal Genes by Fluorescence In Situ Hybridization (FISH)

The analysis of the chromosomal location of the major ribosomal genes (rDNA regions containing the genes for the 18S, 5.8S, and 28S rRNAs) showed a striking inter-specific variability, revealing an extraordinary dynamics of change in

triatomine genomes without deviations in the chromosome number (Panzera et al. 2012, 2014, 2015; Pita et al. 2013, 2016a, 2020; Hieu et al. 2019; Villacís et al. 2020). In 67 triatomine species hitherto analyzed (Table 2), excepting *T. infestans*, the ribosomal genes have one or two chromosome loci per haploid genome (Panzera et al. 2012, 2014), similar as reported in almost all hemipteran species (Grozeva et al. 2015). Triatomines showed four rDNA chromosomal patterns: (a) on one sex chromosome (always X chromosome in XY system or in multiple Xs) (Fig. 3a), (b) on two sex chromosomes (X plus Y chromosomes in XY systems, two Xs or X plus Y chromosomes in multiple sex systems) (Fig. 3b), (c) on one autosomal pair (Fig. 3c), (d) on the X chromosome plus one autosomal pair (Fig. 3d).

As observed with other chromosomal markers, there are remarkable differences in the location of ribosomal genes between the Rhodniini and Triatomini tribes. All Rhodniini species (genera *Psammolestes* and *Rhodnius*) present the rDNA loci on one (X chromosome) or both (X and Y) sex chromosomes (Table 2). On the contrary, in the Triatomini tribe the four patterns described above are observed, being by far the autosomal pattern the most frequent, recorded in species with different chromosome numbers and sex chromosome systems (Table 2). For this reason, this autosomal pattern could be considered ancestral for Triatominae, or at least for the Triatomini tribe (Pita et al. 2016a). Nevertheless, it has been also proposed as the ancestral state for the Heteroptera order (Grozeva et al. 2015). Thus, the relocation of the ribosomal clusters from autosomes to one or two sex chromosomes would be a derived state (apomorphic character). Considering that rDNA loci on one or two sex chromosomes are observed in all Rhodniini species as well as phylogenetically distant Triatomini groups (*Dipetalogaster*, *Eratyrus*, *Mepraia*, *Panstrongylus*, and several *Triatoma* species), it is most likely that the transfer of rDNA loci from autosomes to sex chromosomes has occurred several times during the evolution of this subfamily.

The great reorganization on the chromosomal location of the ribosomal loci, without changes in the chromosome number, suggests that ectopic recombination and/or transposition processes followed by amplifications are involved in the relocation of rDNA loci in triatomines (Panzera et al. 2012; Pita et al. 2013). Probably inter-chromosomal mobility of rDNA loci by transpositions events is facilitated by the presence of transposable elements adjacent to the ribosomal genes (Schubert and Wobus 1985; Zhang et al. 2008). An important fraction of the triatomine genome is composed of transposable elements (Gilbert et al. 2010; Mesquita et al. 2015; Fernández-Medina et al. 2016; Pita et al. 2017c; Castro et al. 2020), including a retrotransposon inserted inside the 28S rDNA sequence in *R. prolixus* (Jakubczak et al. 1991). An rDNA mobility associated with transposable elements was also suggested in other insect groups (Cabrero and Camacho 2008; Nguyen et al. 2010; Cabral-de-Mello et al. 2011).

The transfer of the rDNA loci from autosomes to sex chromosomes has important genetic consequences since it drastically modifies the dynamics of recombination and gene flow, and therefore the genetic differentiation and speciation processes (Sætre et al. 2003). These changes are even more drastic when they involve achiasmate sex chromosomes, as observed in triatomine males (Pita et al. 2016a). The reduced rate of recombination between the sex chromosomes would allow the rapid

emergence of genetic barriers to gene flow and the accumulation of genetic incompatibilities, promoting the divergence of triatomine species as has been observed in Lepidoptera (Šíchová et al. 2013).

The chromosomal position of the major ribosomal clusters is a species-specific character in triatomines, in spite of the intra-specific variability reported in *T. infestans* and *R. ecuadoriensis* (Panzera et al. 2012, 2014; Pita et al. 2013, 2016a). At the evolutionary level, there is a strong tendency for species with a recent common ancestor to have ribosomal genes in the same chromosomal position. Considering the autosomal position as the ancestral character for triatomines, Pita et al. (2016a) suggested that the movement of rDNA loci from autosomes to sex chromosomes rapidly established reproductive barriers between divergent lineages. Because the movement of rDNA loci from autosomes to sex chromosomes has occurred independently several times in unrelated triatomine groups, it is necessary to compare the evolutionary relationships obtained with this rDNA marker with other phylogenetic traits, such as nuclear and mitochondrial gene sequences. Based on these analyses, Pita et al. (2016a) proposed a regrouping of species that constituted several subcomplexes of South American *Triatoma lineage*, supported by the recent systematic revision of Triatominae (Monteiro et al. 2018).

As reported with the C-banding technique, *T. infestans* presents a wide genome diversity revealed also by an rDNA polymorphism in the number of rDNA loci (one to four per haploid genome) and in their chromosomal position (on one or two autosomal pairs, X chromosome only, X chromosome plus one to three autosomal pairs) (Panzera et al. 2014) (Fig. 4). *Triatoma infestans* has the highest number of ribosomal loci detected in Cimicomorpha infraorder, only exceeded in Auchenorrhyncha (Hemiptera) by the seven loci recently described in the cicadomorphan *Membracis foliatafasciata* (Anjos et al. 2019). The high number of ribosomal loci in *T. infestans* could be explained by the mechanism proposed by Dubcovsky and Dvorak (1995) for the spread of ribosomal loci in plants. This mechanism consists in the transposition of a few rRNA clusters to new chromosome locations, their amplification originating new NORs, and the elimination of the old NORs. The other report about intraspecific polymorphism of rDNA location involved different populations of *R. ecuadoriensis* from Peru and Ecuador (Pita et al. 2013). Molecular data suggest that these populations could represent incipient species (Abad-Franch and Monteiro 2005; Villacís et al. 2017).

7.4 Genomic In Situ Hybridization (GISH) and DNA Probes

Among insects with holocentric chromosomes, chromosomal analyses using total genomic DNA, isolated repetitive fractions, or specific repetitive DNAs (excepting rDNA and histone clusters) have been applied in a few species (Yoshido et al. 2006; Bressa et al. 2009; Bardella et al. 2014a; Pita et al. 2014, 2017b, 2018; Lukhtanov et al. 2015; Manicardi et al. 2015b; Anjos et al. 2016; Gallo et al. 2017).

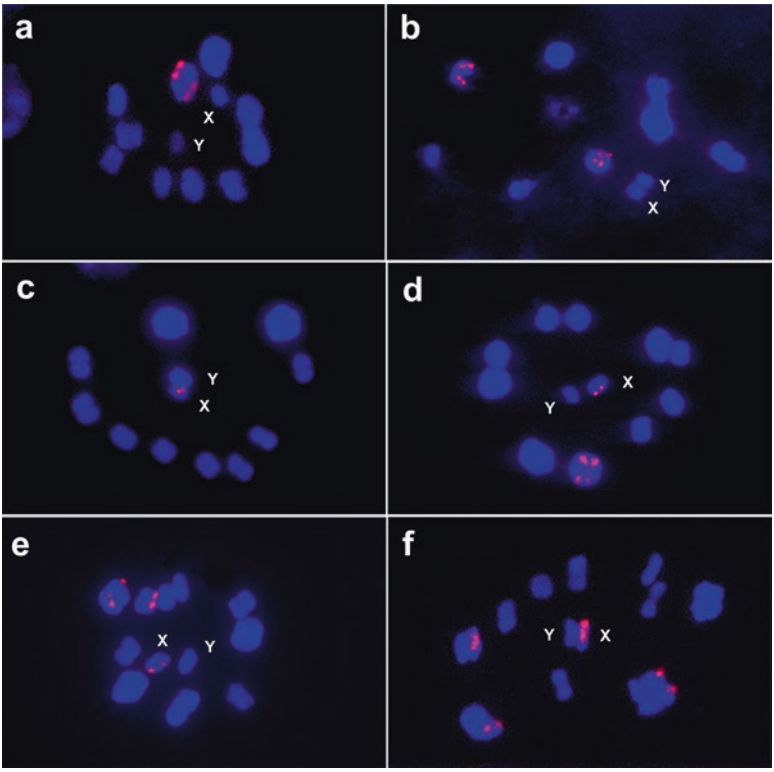

Fig. 4 Intraspecific variability in the chromosome location of the 45S rDNA clusters in *Triatoma infestans* (2n♂= 20A + XY) revealed by FISH. First meiotic division (diakinesis or metaphase I). The rDNA signals are located on: (**a**) one autosomal pair, (**b**) two autosomal pairs, (**c**) only X chromosome, (**d**) X chromosome plus 1 autosomal pair, (**e**) X chromosome plus 2 autosomal pairs, (**f**) X chromosome plus 3 autosomal pairs

In triatomines, we analyzed the chromosomal differentiation of repeated sequences by GISH (genomic *in situ* hybridization) using four *Triatoma* species genomic DNA as probes (two from South American and two from North American lineages). GISH assays were performed on the chromosomes of the species that were used as genomic probes (Self-GISH) as well as on other eleven species belonging to six genera of the Triatomini and Rhodniini tribes (Pita et al. 2014, 2017b). In Triatomini, the results showed that autosomal heterochromatic regions are constituted by species-specific DNA repeats, most probably satDNA families. This suggests that chromosome diversification involved a differential amplification of diverse families of repeated DNA sequences. A very different situation is observed in the Y chromosome of the Triatomini tribe. Although this chromosome is also composed by accumulated repetitive sequences, these sequences are highly conserved among all Triatomini species analyzed (14 species from five genera) (Pita et al. 2014, 2017b).

Employing Illumina sequencing and the RepeatExplorer pipeline, we showed that in the *T. infestans* genome the repetitive DNA fraction, also called repeatome, constitutes around 40% of total genome (Pita et al. 2017c). By far, the most frequent repetitive DNAs are the satellite DNA (satDNA) sequences (30% of the genome), followed by transposable elements (6%). Although we characterized 42 satDNA families, the five most abundant families represent about 90% of the total satDNA. There is a wide range of variation in the repeat unit (monomer) length of each family (between 4 and 1000 bp), although most of the satDNA families (34 of 42) have a repeat unit below 120 bp. By FISH, we determined that eleven satDNA families (those with more than 0.03% of the genome) are located in heterochromatic and euchromatic regions, both in autosomes and sex chromosomes. Bardella et al. (2014a) previously reported three of them on heterochromatic chromosomes.

Another satDNA family identified by the RepeatExplorer pipeline was a telomeric repeat (TTAGG)n, considered ancestral for arthropods (Frydrychová et al. 2004). Considering that the haploid genome content in *T. infestans* is 1.487 Mb (Panzera et al. 2010) and their chromosome number (2n = 22), the average telomeres length motifs in each chromosome end would be almost 18 kb long. By FISH we confirmed the presence of this telomeric repeat in triatomine species of three different genera (Pita et al. 2016b), contradicting the accepted hypothesis that the ancestral insect telomere motif has been lost in evolutionarily advanced heteropterans (Cimicomorpha and Pentatomomorpha infraorders) (Frydrychová et al. 2004; Grozeva et al. 2011; Mason et al. 2016). Recently, Grozeva et al. (2019) confirm the presence of (TTAGG)n repeat in two reduviid species of the genus *Rhynocoris*, considered as a basal family of Cimicomorpha.

Rhodnius prolixus genome is the only triatomine genome assembled hitherto. A total of 15,456 protein-coding genes and 738 RNA genes were identified. TEs were determined to encompass about 5.6% of the total genome (Mesquita et al. 2015). However, Castro et al. (2020) showed that the total amount of TEs in *Rhodnius* genomes, including *R. prolixus*, is three to four times higher (19% to 23.5%) than the original quantification performed by Mesquita et al. (2015). Using bioinformatics pipeline based on low-coverage sequencing data, Montiel et al. (2021) found that the satDNA sequences only represents 8% of the total genome in *R. prolixus*, composed by 39 satDNA families. A comparative search between *T. infestans* and *R. prolixus* genomes have shown that only four satDNA families were shared between both species (Pita et al. 2018). In addition, monomers of three families showed mutations (dimerization and indels), which indicates the large differentiation between *T. infestans* and *R. prolixus* repeats. Chromosome localization was also determined for the four shared satDNA families, depicting euchromatic localization through the entire genome. Hence, contrary to what it is observed in *T. infestans*, satDNA is not the main fraction of the repetitive sequences in *R. prolixus*.

7.5 Y Chromosome in Triatominae

All Triatomini and Rhodniini species currently analyzed by classical C-banding showed a heterochromatic Y chromosome. However, fluorochrome banding (Bardella et al. 2010, 2014a; Bardella et al. 2016a, b), GISH (Pita et al. 2014, 2017b), and satellite DNA analyses (Pita et al. 2017c, 2018) reveal a striking differentiation in the sequence composition of the Y chromosome between both tribes. In Triatomini, Y chromosome is DAPI+/CMA_3^-, mainly composed by A+T rich repeated DNA sequences (Bardella et al. 2016a). GISH analysis of the three Triatomini lineages (including fifteen species of five genera) revealed that Y chromosome is highly conserved among all Triatomini species and it is constituted by highly repeated sequences (Pita et al. 2014, 2017b). The former described results employing RepeatExplorer pipeline on next-generation sequencing reads, followed by the characterization and isolation of the main satellite DNA families revealed that the *T. infestans* Y chromosome is mainly constituted by two DNA repeats: TinfSat01-33 and TinfSat03-4/$(GATA)_n$ repeats (Pita et al. 2017c). Unpublished data using simple sequence repeats (SSR) as DNA probes demonstrated that (GATA)n repeats are the only satDNA family that is shared on the Y chromosome among Triatomini species. These $(GATA)_n$ repeats probably represent an ancestral character of this tribe. Exceptionally, in four species the Y chromosome also includes a CMA_3+ region (Bardella et al. 2016a), which coincides with the localization of ribosomal clusters (Panzera et al. 2012, 2015). Considering that Y chromosomes carrying CMA_3+ regions are present in evolutionarily distant species (*Eratyrus cuspidatus*, *T. sordida* Argentina, *T. matogrossensis* and *T. vitticeps*), we concluded that this trait is an apomorphic character for Triatomini tribe (Bardella et al. 2016a). Contrary to what was observed in Triatomini, the heterochromatic Y chromosome in the Rhodniini tribe did not exhibit DAPI or CMA_3 positive regions, including species with Y chromosome carrying ribosomal clusters (Bardella et al. 2016a). GISH and satDNA probes analyses also suggested that the Y chromosome of Rhodniini is constituted by other types of DNA sequences different from those found in Triatomini (Pita et al. 2014, 2018).

7.6 X Chromosome in Triatominae

Analyses by GISH and chromosome painting reveal that, as observed on the Y chromosome, the X chromosomes of Triatomini and Rhodniini tribes present substantial differences in their repetitive sequences. In Triatomini, unlike the Y chromosome, the X chromosomes exhibit an extensive variability (revealed by different techniques, such as C and fluorochrome bands and FISH with different DNA probes). This wide variation on the X chromosomes is not reported in other heteropteran groups. In most Triatomini species, the X chromosomes are euchromatic without fluorescence regions, similar to autosomal euchromatin. However, in other not

closely related species and with different number of X chromosomes, the X chromosomes have heterochromatic regions either DAPI or CMA_3 positive (Bardella et al. 2016a). In some *Triatoma* species of the South American lineage with 2n = 22 (20A + XY), such as *T. delpontei*, *T. platensis,* and *T. infestans* (Andean group), the heterochromatic regions of the X chromosome have similar repeated sequences to those of the Y chromosome and to the heterochromatic autosomal regions (Pita et al. 2014, 2017b). In some *Triatoma* species of the North American lineage with 2n = 23 (20A + X_1X_2Y), such as *T. barberi* and *T. ryckmani* (Fig. 1i), one or two X chromosomes presented repeated sequences similar to the Y chromosome, but different from the autosomal heterochromatin (Bardella et al. 2016a; Pita et al. 2017b). CMA_3 positive regions are frequently associated, but not always, with ribosomal clusters (Bardella et al. 2016a). Chromosome painting using both X chromosomes of *M. spinolai* as DNA probes reveals that the euchromatin of the Triatomini X chromosome contains dispersed repeated sequences that are similar to the ones located in the autosomal euchromatic regions (Pita et al. 2017a). Considering that the heterochromatic X chromosome is also observed in evolutionarily distant Triatomini species with different sex mechanisms, its occurrence is probably due to convergent evolution. Thus, the ancestral X chromosome should have been similar to the euchromatic autosomes without C-heterochromatic regions or fluorochrome banding.

The X chromosome of Rhodniini presents very different characteristics as compared to Triatomini. All analyzed species of *Rhodnius* and *Psammolestes* have an XY/XX system (Table 1), with the X being always euchromatic without fluorescence signals, even though they always carry the ribosomal clusters (Panzera et al. 2010, 2012; Bardella et al. 2016a). The hybridization signals with Triatomini X chromosomes probes are small and scattered across all euchromatin, without specific regions including the X chromosome (Pita et al. 2017a).

In summary, the cytogenetic evidence shows that both the autosomes and the sex chromosomes of the Rhodniini and Triatomini tribes present significant differences in the repeated sequences that constitute their genomes. Considering the controversy about the monophyletic (Hypša et al. 2002), paraphyletic (Hwang and Weirauch 2012), or polyphyletic (Schofield 1988) origin of the Triatominae subfamily, the substantial chromosomal and genomic differences between Triatomini and Rhodniini tribes offer us new tools to address this problem. Molecular analysis using fossil-calibrated relaxed clock models estimated that the divergence of the Triatominae from their predatory reduviid ancestors ranges from 35 to 40 million years ago (Hwang and Weirauch 2012; Ibarra-Cerdeña et al. 2014; Justi et al. 2016; Zhang et al. 2016; Monteiro et al. 2018). This long period seems to be enough to generate the substantial genomic differences found between the two triatomine tribes. Comparative analyses of repetitive chromosome sequences among triatomines with putative sister reduviid groups, particularly Stenopodainae and Reduviinae (*Ophistacidius* and *Zelurus),* may help to clarify the origin of the Triatominae subfamily. Chromosomal information of these subfamilies is limited to a little more than ten species, presenting autosome numbers that vary between 20 and 26 and several sex chromosome systems with multiple X chromosomes

(Ueshima 1979). For example, *Zelurus femoralis* presented a diploid chromosome number of 22 (20 autosomes plus XY/XX) and heterochromatic Y chromosome, similar to Triatomini tribe (Poggio et al. 2013b). Comparative analysis among triatomines with sister reduviid groups using complete mitochondrial genomes could offer another alternative to elucidate the controversial origin of kissing bugs (Pita et al. 2017d).

Perspectives and Challenges

In order to have a complete picture of the evolution of the Triatominae subfamily, it is essential to analyze the chromosome composition of all species belonging to the smaller tribes (Alberproseniini, Bolboderini, Cavernicolini), the 13 endemic species from Asia (seven *Triatoma* and six *Linshcosteus*), and the evolutionarily close reduviid groups. Chromosome information should not be limited to the mere description of the chromosome number but should also include other cytogenetic and genomic characteristics such as the genome size and the molecular composition of the different repeated fractions.

Considering that triatomine species exhibit large differences in their nuclear genome contents but with similar chromosome numbers, it is evident that these differences are due to variations in the amount of non-coding DNA, especially satDNA and transposons. As have been seen in the Triatomini tribe, the divergence between the species has been accompanied by changes in the quantity and types of satDNA sequences. However, in Rhodniini, the amount of heterochromatin is substantially lower than in Triatomini, so the role of satDNA as a differential factor between species may be less important. The genome sequencing of other Rhodniini species will allow to determinate the role of other repetitive sequences involved in the genome differentiation process.

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Embryonic Development of the Kissing Bug *Rhodnius prolixus*

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Abstract The dissemination of neglected tropical diseases that is performed by insect vectors is highly dependent on processes that take place during embryogenesis, herein defined as the process from egg deposition until hatching. To efficiently maintain the next generation of infective animals, eggs that are laid by the Chagas disease vector *Rhodnius prolixus* contain all of the molecular information that is required for embryonic development. Several morphogenetic processes take place during this time, after which a miniature of the adult emerges from the egg. Here, we present the current knowledge on the embryonic development of *R. prolixus*. Firstly, we present a historical overview of *R. prolixus* embryology from the earliest studies in the first half of the twentieth century to present. Then, we discuss how recent advances in functional genomics might foster new discoveries related to the molecular control of *R. prolixus* embryology and its interface with vector population control.

Keywords Oncopeltus · Dorsal · BMP · Gene regulatory network · Phylogeny · Evo-Devo

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1 General Observations of Insect Development

Insects are considered to be the most pervasive class of animals, since approximately 75% of all animal species belong to this group (Grimaldi and Engel 2005). Their adaptability to different ecological niches requires, among other aspects, different modes of embryonic development, in addition to the production of specialized protective structures that cover the embryo. For instance, changes in eggshell structure have been suggested to be essential for insect adaptation to the terrestrial habitat (Zeh et al. 1989; Church et al. 2019). Several authors have described this morphological variability, and thus we refer the reader to these publications for further details about general insect embryology (Counce and Waddington 1972; Anderson 1973; Davis and Patel 2002; Roth 2004).

In this chapter, we focus on studies concerning the embryonic development of *Rhodnius prolixus,* which is a prominent vector of Chagas disease in the Americas (reviewed in de Fuentes-Vicente et al. 2018). *Rhodnius prolixus* is a blood-sucking insect belonging to the Triatominae subfamily (genus Reduviidae) that transmits the protozoan *Trypanosoma cruzi*. The protozoan aetiologic agent of Chagas disease is spread through insect faeces during the blood meal. It has been estimated that 6–7 million people are chronically infected by *T. cruzi,* with 50–60 million at risk of contracting the disease (Schofield et al. 2006; Coura and Dias 2009). Chagas disease comprises a spectrum of symptoms including chronic cardiac, digestive and neurological and mixed disorders. Over the past 50 years, the use of insecticides, chiefly pyrethroids, has proven successful at suppressing insect vector populations, and therefore the transmission of the disease, in different South American areas. However, concern is raised by the development of resistance or tolerance to the insecticides, which leads to the selection of resistant insects and the failure of strategies for vector population control. The limited variety of insecticides that are available, together with toxicological concerns, has resulted in the development of novel strategies that might be used in combination with pesticides or as possible alternatives once resistant vector populations appear (Traverso et al. 2017). The sterile insect technique has been broadly used to eradicate or control insects of agricultural or medical relevance, including *Cochliomiya hominivorax* (Skoda et al. 2018) and *Ceratitis capitata* (Juan-Blasco et al. 2014). However, this technique relies on the mass rearing, sterilization and release of insects, which compete with the native mates in the infested areas, causing a rapid reduction of the endemic population. Although it is very effective, this strategy might prove cumbersome for *R. prolixus* given that sterile males and females can still transmit the disease if they are infected, and the mass rearing would require massive facilities and significant costs. The recent development of the CRISPR/Cas9 system and its derivatives like the Mutagenic Chain Reaction for genome editing might help overcome these limitations and allow the design of novel approaches for the control of triatomine populations (Gantz and Bier 2015). It is conceivable, for example, that the release of fewer genetically engineered insects might allow the introduction and spread of alleles providing resistance to *T. cruzi* infections or causing sterility or lethality in the

target population. Albeit promising, such strategies imply a deeper knowledge of vector biology. Thus, unravelling the genetic and molecular underpinnings of embryogenesis in this insect will not only help shed light on evolutionarily conserved mechanisms, but it might also pave the way for the development of novel control strategies that can prevent the spread of Chagas disease. During the embryonic period, the insect cannot disperse; thus, processes taking place during this period may be interesting targets for biological control. Another basic but noteworthy aspect of embryonic development concerns its dependence on maternally supplied signals and molecules. *Rhodinus prolixus* eggs are laid with a great amount of molecules that are provided by the mother such as nucleic acids, proteins, lipids and carbohydrates (Atella et al. 2005). Thus, oogenesis and embryogenesis are tightly linked, implying that strategies aimed to control embryonic development may also be designed to target oogenesis.

In contrast to the fruit fly *Drosophila melanogaster* and the beetle *Tribolium castaneum*, which are two holometabolous model systems that undergo larval and pupal stages, *R. prolixus* is a hemimetabolous insect. *R. prolixus* embryos develop into first-instar nymphs, which already resemble the adult morphology after hatching from the eggshell and undergo five nymphal stages before moulting into fertile adult insects (Fig. 1).

While *R. prolixus* physiology has been largely investigated, studies of embryonic development are still scarce. This chapter aims to highlight the seminal studies that have been performed at the beginning and middle of the last century that described the embryonic development of *R. prolixus*. In addition, we review studies that have been performed in the past decade using modern molecular and genomics approaches and have started to reveal the mechanisms underlying *R. prolixus* embryogenesis. We hope that by the end of the chapter, the reader is convinced that embryonic development is an essential but neglected process of kissing bug biology that will help shed light on insect evolution and provide promising targets for novel vector control strategies.

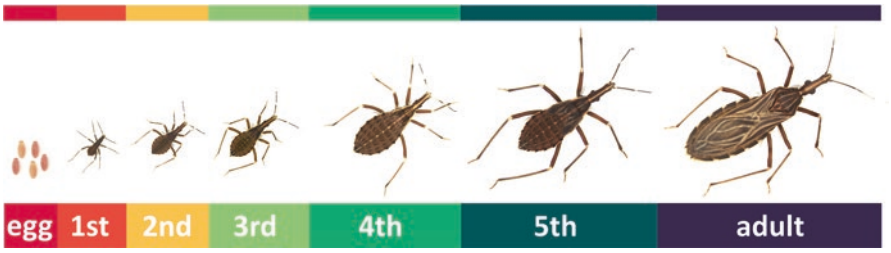

Fig. 1 *Rhodnius prolixus* life cycle. *Rhodnius prolixus* eggs and the nymphal and adult stages. Blood feeding is essential for moulting in all free-living stages. During embryogenesis, a clear variation in egg colour is observed, as freshly laid eggs are reddish when compared with the late embryonic developmental stages

2 Oogenesis and Embryogenesis in Model Species and Their Relevance to *R. prolixus* Embryology

Before the egg is laid, an extensive process of egg development, termed oogenesis, takes place inside the mother. Oogenesis and embryogenesis are highly intertwined since improper oogenesis, lack of oocyte growth or chorion formation dramatically affect embryogenesis in several insect species (Lynch et al. 2010). Oogenesis starts by defining the germline versus accessory somatic stem cells. The generation and amount of germline stem cells are either continuous, as shown in adult females of *D. melanogaster*, or specified during early stages of development, such as during *R. prolixus* nymphal stages (Telfer 1975; Brito et al. 2018).

Ovaries of species belonging to basal insect groups are panoistic, which means that the egg chamber is formed by the oocyte and an external layer of follicle cells, while it lacks accessory germline nurse cells (Büning 1994). Examples of panoistic ovaries are those of orthopterans such as *Gryllus bimaculatus* and *Thermobia domestica* (Ewen-Campen et al. 2013; Tworzydlo et al. 2014). Nurse cells appeared later in insect evolution and are considered a feature of meroistic ovaries. Two types of meroistic ovaries can be observed: the telotrophic and the polytrophic types. In the former, the nurse cells remain in the tropharium and are connected through trophic cords to the developing oocyte. By contrast, in the polytrophic type, the nurse cells are hosted in the egg chamber, where they keep nourishing the oocyte until its mature stage. *Dorsophila. melanogaster* is an example of a meroistic polytrophic ovary, while *R. prolixus* displays a meroistic telotrophic ovary type. An evolutionary correlation between types of embryogenesis and oogenesis has been previously suggested (Davis and Patel 2002; Lynch et al. 2012), for example., species with meroistic polytrophic ovaries display faster embryogenesis of the long-germ type, while species with panoistic and meroistic telotrophic ovaries show slower embryogenesis of the short-germ type (Fig. 2). A detailed description of the *R. prolixus* meroistic telotrophic ovary is provided here.

Adult *R. prolixus* females display two ovaries and each ovary is formed of 6–8 ovarioles that are encapsulated by a basal lamina and surrounded by a tracheole-rich sheath (Huebner 1981b; Lutz and Huebner 1981). A pedicel connects all of the ovarioles to the oviduct and constitutes the path taken by the mature eggs during oviposition. As in *D. melanogaster*, the ovariole resembles an assembly line with newly formed egg chambers sequentially budding from the tropharium, which is a lancet-like structure found at the very anterior end and moving towards the posterior region of the ovary (King 1970; Huebner and Anderson 1972; Lutz and Huebner 1980). During this process, the egg chambers gradually develop until they turn into a mature chorionated egg. As mentioned here, a remarkable feature of the telotrophic ovaries is that the nurse cells are retained in the tropharium, where they undergo a turnover (Huebner 1981a; Lutz and Huebner 1981). Mitotically active diploid nurse cells are hosted in the anterior tip of the tropharium. These cells provide a reservoir of proliferating cells in zone 1 but are also a source of nurse cells that are transferred to zone 2 and zone 3, where they stop dividing, engage in

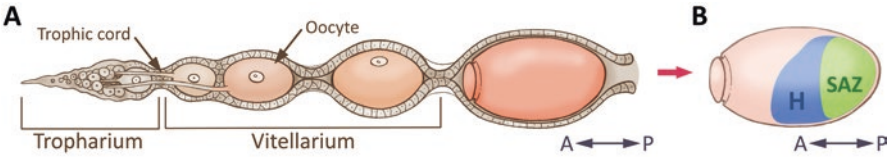

Fig. 2 The *Rhodnius prolixus* ovariole and its relationship to embryonic development. (**a**) A schematic drawing of a single ovariole. The tropharium, the vitellarium, the trophic cords and the oocytes are highlighted. Note the migration of the single haploid nucleus towards a cortical region. At the later stages of oogenesis and during choriogenesis, a clear anterior-posterior axis is evident (A-P), including an anterior cap. (**b**) An *Rhodinus prolixus* egg shortly before gastrulation. As an embryo of the short-germ type, only the head segments (H) are formed early in development in the posterior-ventral region of the egg; the posterior segments are formed as a secondary process from the segment addition zone

endoreduplication and become polyploid. The nurse cells or trophocytes display large nuclei in the latter zones and apparent nucleoli indicating intense biosynthetic activity. In addition, the cells are organized in finger-like projections emanating from the periphery of the tropharium, where the cells form a syncytium. This architecture allows extensive communication between the cells, as it is also observed in *D. melanogaster* and other species, where the nurse cells remain interconnected through cytoplasmic bridges known as ring canals after each mitotic division (reviewed in Bastock and St Johnston 2008). However, in contrast to *D. melanogaster*, where oocytes are continuously produced from germline stem cells, *R. prolixus* displays a limited number of oocytes blocked in the prophase of the first meiotic division and grouped in the posterior region of the tropharium. In this species, the oocytes are produced from germline stem cells that are restricted to the nymphal stages and are not maintained in the adult (Telfer 1975; Brito et al. 2018). The oocytes are sequentially encapsulated by the follicle cells, thus forming the budding egg chambers. Since the nurse cells remain in the tropharium, nutrients and possibly RNAs are transported to the growing oocytes through cytoplasmic bridges known as trophic cords, which allow the egg chambers to remain connected to the tropharium throughout the vitellogenic stage (Huebner 1981a; Harrison and Huebner 1997; Melo et al. 2000). Upon oocyte growth during vitellogenesis, the trophic cords increase in diameter and can reach lengths of close to 1 mm (Huebner 1984). At the beginning of choriogenesis, the trophic cords are severed and the oocyte starts to be enclosed in a hard-impermeable shell, which is the chorion. As in *D. melanogaster*, *the R. prolixus* egg displays apparent anterior-posterior (AP) and dorsal-ventral (DV) axes, whereby the anterior region is marked by the operculum, which is clearly tilted towards the dorsal side. The molecular mechanisms that control axial polarization in this insect species are completely unknown. In *D. melanogaster*, the TGF-α like protein Gurken (Grk) is asymmetrically localized in the early egg chambers, where its accumulation at the posterior end of the oocyte determines the posterior pole of the future embryo (Neuman-Silberberg and Schupbach 1993; Gonzalez-Reyes et al. 1995). Later, during mid-oogenesis, the Grk morphogen is re-localized to the future dorsal-anterior corner of the oocyte together with the

oocyte nucleus, following an extensive reorganization of the microtubule network. In this position, Grk signals the dorsal fate to the adjacent follicle cells (Neuman-Silberberg and Schupbach 1993). Although Grk orthologues have not been found in species other than the drosophilids, the repositioning of the oocyte nucleus is a symmetry-breaking event that marks the dorsal side of the egg in insects as distant as beetles and flies (Neuman-Silberberg and Schupbach 1993; Lynch et al. 2010). Grk is responsible for activating the torpedo/epidermal growth factor (EGF) receptor in a single layer of follicle cells (Roth and Schupbach 1994; Lynch et al. 2010). In *D. melanogaster,* EGF signalling in the dorsal follicle cells generates differences in the ventral follicle cells that later culminate in activation of the Toll pathway in ventral and lateral region of the early embryo, defining the embryonic DV axis. Toll pathway activation, which is mediated by a complex network of proteases and interaction partners, has been reviewed elsewhere (Moussian and Roth 2005). We have shown that Toll pathway elements are expressed during oogenesis in *R. prolixus* and parental knockdown assays reveal an important role in controlling the early aspects of embryonic development (Berni et al. 2014). EGF pathway orthologues have been detected in this species, but their role has not yet been investigated (Mesquita et al. 2015). In *D. melanogaster*, additional maternal determinants that are asymmetrically deposited in the oocyte play critical roles in the establishment of the AP axis (i.e. the Bicoid protein) and in the formation of the germ plasm (i.e. the Oskar protein and mRNA) (Lehmann and Nusslein-Volhard 1986; Driever and Nusslein-Volhard 1988; Ephrussi et al. 1991). None of these genes, however, appears to have corresponding orthologues in the *R. prolixus* genome. Similarly, no evidence of a germ plasm has been found in this species. Not all hemipteran eggs display clear DV asymmetry when laid. For example, *Oncopeltus fasciatus*, the hemipteran species most studied at the embryological level (Lawrence 1969; Lawrence and Hayward 1971; Lawerence and Green 1975; Liu and Kaufman 2004a, b; Angelini et al. 2005; Panfilio et al. 2006; Erezyilmaz et al. 2009a, b; Liu and Patel 2010) does not show a clear distinction between the ventral and dorsal sides at the beginning of embryogenesis. Hence, *R. prolixus* represents an excellent model system to investigate the genetics and evolution of axial polarization in insects of basally branching groups. It is conceivable that if maternally provided novel mRNAs or proteins are responsible for the establishment of the body axes in *R. prolixus*, their transport and asymmetric localization must to a certain extent involve the trophic cords. It will be of great interest to unravel these mechanisms in the future.

After vitellogenesis, the chorionic stages allow the production of the eggshell. The non-pigmented chorion is extremely resistant but allows the transmission of the pink coloration of the vitellum inside the egg. The chorion is composed of seven different layers that form a resistant exochorion and a more internal and softer endochorion (Beament 1946a). Understanding how these layers are placed and produced is fundamental to devise new strategies to introduce pharmacological agents and nucleic acids in the embryo for functional studies. For instance, it was recently shown that the mature eggshell presents channels that can be used for the delivery of pharmacological inhibitors by permeabilization with ethanol (Beament 1946b; Bomfim et al. 2017).

Lastly, the biochemical and molecular bases of *R. prolixus* oogenesis have been studied in more detail during the past years (references in Atella et al. 2005, Alves-Bezerra et al. 2016, Vieira et al. 2018, Brito et al. 2018). One crucial aspect of *R. prolixus* physiology that differs from their plant-feeding relatives *O. fasciatus* and the pea aphid *Acyrthosiphon pisum* is their obligatory blood-feeding behaviour. *Rhodinus prolixus* blood-feeding is required for oogenesis but leads to a great oxidative challenge due to haeme release during blood digestion in the midgut lumen (Oliveira et al. 1999). Several strategies have been developed to deal with this problem among blood-feeding insects (Sterkel et al. 2017). In *R. prolixus*, haeme resulting from the processing of haemoglobin is transported by the haemolymphatic haeme-binding protein (RHBP) to the vitellogenic oocytes, where it is stocked in yolk granules for embryonic development. Impairment of RHBP function does not alter oviposition, but it generates white haeme-depleted eggs that do not develop beyond early embryonic stages (Walter-Nuno et al. 2013). A recent analysis of the role of iron and haeme-related genes in *R. prolixus* showed that the correct development of the embryos depends on an adequate iron delivery during oogenesis, which is performed by maternal ferritin, implicating this protein as the major transporter of iron for growing oocytes (Walter-Nuno et al. 2018). Thus, proper blood digestion and haeme transport to the ovary are essential for oogenesis and embryogenesis.

3 Historical Role of *R. prolixus* Embryonic Development Studies

Between the end of the nineteenth and the first half of the twentieth century, the embryonic development of several insect species, including *R. prolixus,* were described in great detail. Two seminal studies have described the early (Mellanby 1935) and late (Mellanby 1936) embryonic development of *R. prolixus*. At that time, only the development of the nymphal to adult stages had been described for *R. prolixus* (Buxton 1930). Following Mellanby's definition, *R. prolixus* was proven to be an 'admirable experimental animal' for insect physiology. Importantly, this was the first study that investigated the embryonic development of *R. prolixus* in a temperature- and humidity-controlled environment: at 21 °C and 90% humidity, more than 90% of nymphs hatched after 29 days from oviposition. A histological analysis provided details of *R. prolixus* embryogenesis, particularly with the observation of cleavage pattern, blastoderm formation, localization of germ cells at the posterior pole of the egg, mesoderm formation via invagination and overgrowth and endoderm emergence by proliferation from two regions, an anterior and a posterior area (Mellanby 1935). In addition, the author showed that the position of the embryo is reversed in respect to the egg axis as a result of the early invagination and blastokinesis. After invagination is complete, the embryo lays in a superficial dorsal position, with the head of the embryo at the posterior pole of the egg. The head of the embryo is flexed around the posterior pole of the egg so that its extreme anterior

portion is ventral in position. One year later, Hellen Mellanby described the development of the *R. prolixus* embryo from the end of the gastrulation stage until the nymph hatches from the egg. During these germband stages thoracic appendages (legs) start to be distinguished from the maxillary and mandibular outgrowths, and the head and thorax are clearly distinct from the abdomen of the embryo by their different widths. Several aspects of late embryonic development were followed in extreme detail such as the formation of the cuticular layer, formation of the dorsal organ from the serosal embryonic membrane, the central nervous system, the corpus allatum, fore-, hind and midgut, mesodermal derivatives, migration of the germ cells in coeloms of the posterior abdominal segments and, most importantly, a timetable of development at 90% relative humidity. Only 50 years later, another complete investigation of *R. prolixus* embryonic development was performed (Kelly and Huebner 1989). As described by the authors, several manuscripts addressed the process of oogenesis during this period, while embryogenesis was largely neglected (reviewed in Huebner 1984). Most observations of Mellanby (1935) were confirmed by Kelly and Huebner (1989), although particular differences in temperature and humidity led to differences in the time of development. Particularly, Kelly and Huebner (1989) demonstrated some differences in the timing of the events. At higher temperature (26 °C), Kelly and Huebner (1989) observed several events earlier than Mellanby, such as the observation of energids reaching the cortex several hours earlier than previously reported and germ cell identification at earlier stages as a separate population in the posterior region. The site of invagination at the posterior pole of the egg appears as a large region rather than the 'point' described by Mellanby. In addition, Kelly reported a caudal flexure during the process of germ band segmentation that was not observed by Mellanby. Importantly, the embryogenesis processes of several other hemipteran species were analysed during the first and second half of twentieth century, such as *O. fasciatus* by Butt 1949, revealing some common processes between *O. fasciatus* and *R. prolixus*. General hemipteran development has been reviewed by Counce and Waddington 1972 and Anderson 1973, among others.

4 Recent Advances in the Studies of *R. prolixus* Embryonic Development

Following a large gap since Huebner's studies during the 1980s and 1990s, a few groups that were originally interested in the development of holometabolous model species such as the fruit fly *D. melanogaster* and *T. castaneum* started to investigate the embryonic development of *R. prolixus* (Lavore et al. 2012, 2014, 2015; Berni et al. 2014; Souza-Ferreira et al. 2014; Mury et al. 2016; Nunes-da-Fonseca et al. 2017; Brito et al. 2018; Tobias-Santos et al. 2019). Most of the motivation for the establishment of *R. prolixus* as a model system to study embryogenesis can be justified: (1) the existence of seminal studies on embryonic and post-embryonic

development, as well as the physiology of *R. prolixus* by V. B. Wigglesworth, H. Mellanby and the Nobel prize winner Francis Crick, among others (Wigglesworth 1960; Wigglesworth 1965; Lawrence et al. 1972); (2) *R. prolixus* belongs to the largest order of hemimetabolous insects, Hemiptera, which are widespread in different habitats (Panfilio and Angelini 2018); (3) the availability of the *R. prolixus* genome sequence (Mesquita et al. 2015); (4) the possibility to perform functional studies, for example, gene knockdown via RNA interference (RNAi). In the case of *R. prolixus* and several other insect species, the knockdown effect of double-stranded RNA (dsRNA) is observed in the next generation upon mother injection or parental RNAi (pRNAi) (Fig. 3) (Bucher et al. 2002; Lavore et al. 2012; Paim et al. 2013; Berni et al. 2014; Tobias-Santos et al. 2019). In the following section we describe how the establishment of these new techniques and the comparison of *R. prolixus* embryonic development with other insects have opened new avenues in triatomine research.

Like many other insects, *R. prolixus* embryonic development starts after fertilization. The *R. prolixus* egg is fertilized prior to oviposition by the passage of sperm through one of the micropyles located near its anterior cap region, inside the oviduct. Oviposition defines the beginning of embryonic development, and the reader is referred to Berni et al. 2014, Nunes-da-Fonseca et al. 2017 for further information about detailed staging of kissing bug embryogenesis. An embryonic staging system is summarized in Fig. 4 and is based on the description of Berni et al. 2014. This embryonic staging system was performed at 28 °C and 70–80% relative humidity, which is the temperature commonly used by current *R. prolixus* researchers and facilities, but this temperature is higher than the one adopted in previous studies from Mellanby (1935) and Kelly and Huebner (1989).

Rhodinus prolixus eggs contain large amounts of yolk. DV polarity of the *R. prolixus* egg is observed immediately after oviposition due to the tilted position of the anterior cap (Fig. 4, Stage 1). During the first 6 h after egg laying (AEL) nuclear division without cytokinesis takes place in the interior of the egg, where a syncytium that is characterized by a common cytoplasm is observed. In the next 6 h AEL (6–12 h) (Fig. 4, Stage 1), the nuclei start their migration to the periphery, where they will be surrounded by membranes, through the phenomenon of cell formation (cellularization). This stage is called uniform blastoderm (Stage 1), where cells are uniformly dispersed at the egg surface. At stage 2a (12–18 h AEL) two populations become morphologically evident. These include the larger anterior-dorsal cells, which will presumably differentiate into the extraembryonic cells of the serosa, and the ventral-posterior cells constituting the embryonic tissue and the amnion (see also, Berni et al. 2014). The embryonic rudiment plus amniotic rudiment correspond to the germ rudiment (GR-early germ band); this is evident at stage 2a. During this blastoderm period (18–24 h) the not yet invaginated ventral GR is visible and further distinct from the serosal cells (Stage 2b). A group of condensed cells that was previously suggested to be germ cells by Mellanby 1935 is visualized at the posterior region of the egg. At approximately 24 h AEL (Stage 3a) gastrulation is initiated with the invagination and inward proliferation of GR cells on the dorsal side and by the movement of the anterior region of the GR towards the posterior

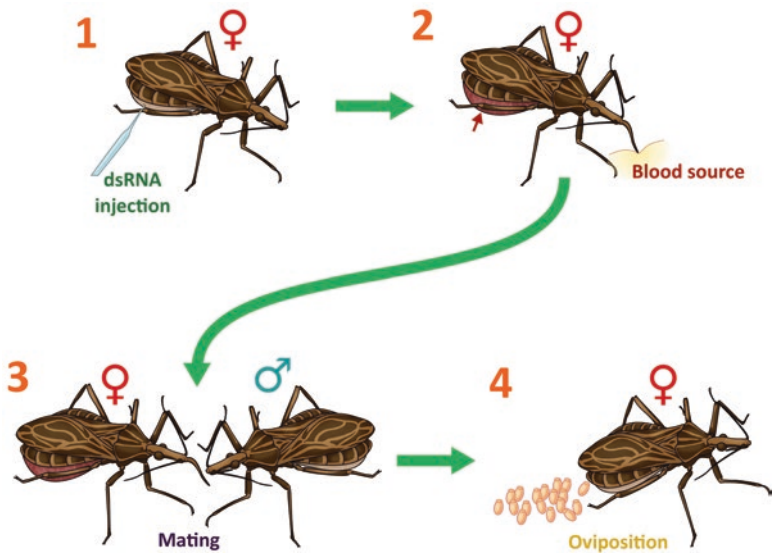

Fig. 3 Parental RNAi method. (1) Adult unfed or starving females are injected with dsRNA against a specific gene of interest. (2) Blood-feeding is performed using vertebrate blood either directly to the animal or using artificial blood feeding. (3) Mating is performed if the female was virgin and had not mated before. (4) Approximately 7 to 10 days after blood feeding, oviposition starts and approximately 40 eggs are laid by each female upon a single gonadotrophic cycle. Eggs are collected and the knockdown efficiency for the gene of interest is evaluated, in addition to other parameters such as hatching rate and morphology after fixation if lethality is observed. The blood-feeding can be repeated several times and depending on the gene, a similar knockdown effect can still be observed after several cycles

pole (Stage 3b); this process is called immersion anatrepsis. Anatrepsis leads to the formation of the amniotic cavity, and germ band extension starts while the cephalic region stays at the posterior region of the egg (Fig. 4, Stage 4). Importantly, by the end of gastrulation the embryo and egg AP axes are not coincident but rather inverted in relation to each other. The same phenomenon occurs with the embryonic and egg DV axis, which are also not equivalent at that stage. Interestingly, immersion anatrepsis seems to be a conserved feature of hemipteran development (Panfilio et al. 2006; Panfilio 2008). In another hemipteran species, *O. fasciatus,* the posterior of the embryo is observed at the ventral side of the egg and not at the dorsal side of the egg as in *R. prolixus*, suggesting differences in the invagination process in both species. Germ band extension and morphological segmentation start to be evident at the anterior region (36 h, Stage 3B) and head/gnathal and thoracic appendages are visible at stage 5 (48 h AEL). Abdominal segmentation is clear at 60 h (Stage 6) and Stages 7, 8 and 9 are characterized by appendage growth and the immersion of the embryonic posterior regions into the yolk and germ band retraction, particularly at Stages 8 and 9 (Stages 7 to 9, 72 to 108 h AEL). From Stage 8 to 10, the embryo undergoes katatrepsis, which is a movement of the embryo emerging from the yolk by performing a 180° backwards rotation. After katatrepsis, the axes of the embryo

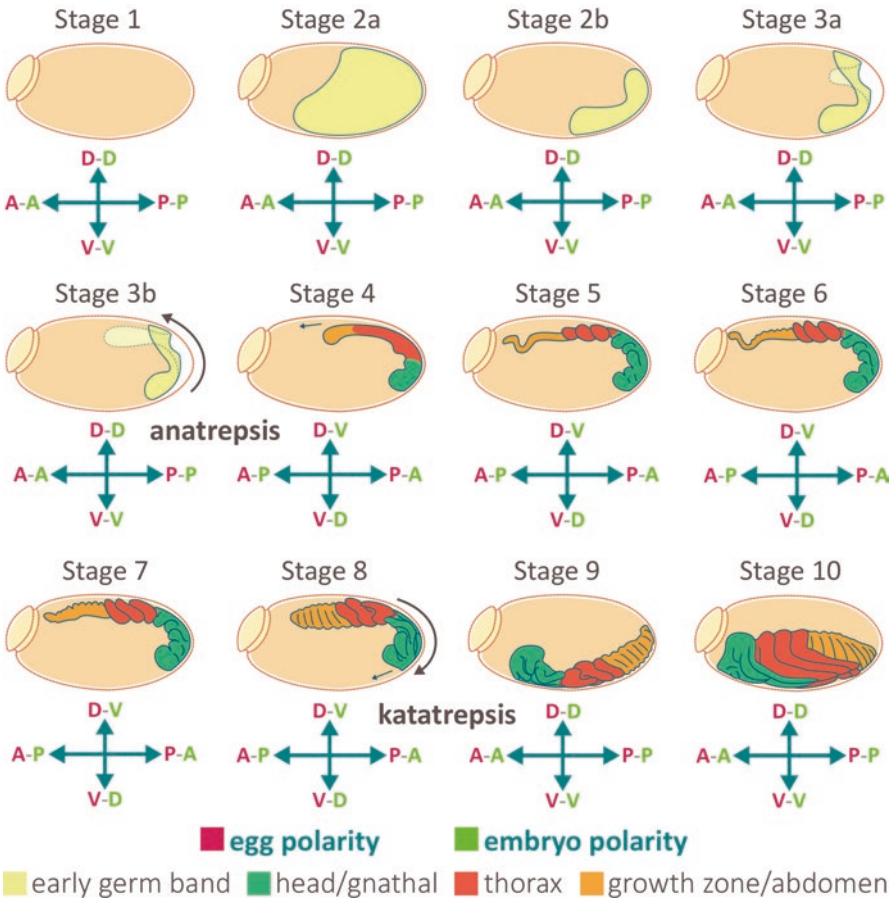

Fig. 4 Developmental stages of the kissing bug *R. prolixus*. The developmental features defining each of the ten embryonic stages have been published by Berni et al. (2014), Nunes-da-Fonseca et al. (2017). These stages can be defined by clear morphological distinctions. Stage 1: Embryonic cells are located uniformly in the periphery of the egg. Stage 2a: At stage 2a, the germ rudiment (embryo plus amnion) is represented in yellow at the posterior ventral region of the egg. Stage 2b: The ventral posterior germ rudiment is further concentrated at the posterior region. Stage 3a: The beginning of the posterior invagination process. Stage 3b: Posterior invagination takes place and the posterior region moves towards the anterior dorsal side of the egg. Stage 4: Germ band elongation and the head remains at the posterior region. The axes of the embryo and the egg are distinct. Stage 5: Distinction between anterior and posterior segments becomes more evident. Stage 6: Thoracic and gnathal appendage growth. Stage 7: Beginning of germ band retraction. Stage 8: Germ band retraction with further differentiation of the abdominal segments. Stage 9: Katatrepsis movement re-establishes the coincidence between embryonic and egg polarity. Stage 10: Dorsal closure is essential for embryonic hatching

and the egg are now coincident, and the head becomes located at the anterior region of the egg close to the cap. Similar movements of katatrepsis are observed in other hemimetabolous insects and require the presence of the serosal membrane as previously described (Panfilio 2008, 2009). The method described by Berni et al. 2014 allows fixation and embryonic observation of most developmental stages with the exception of very early pre-blastoderm stages (e.g. 0–3 h) and very late stages (Stages 9 and 10) when cuticle secretion due to chitin synthesis might impair fixation and staining (Souza-Ferreira et al. 2014).

Although the fixation method and staging system that was published by Berni et al. 2014 helped to establish a framework for comparative embryological studies of Evolutionary Developmental Biology (Evo-Devo) in an important insect species for Latin American researchers (Marcellini et al. 2017), the lack of a reliable method for the embryonic detection of mRNA (in situ hybridization) impaired the detailed analysis of embryonic phenotypes. Recently, Nunes-da-Fonseca et al. 2017, Tobias-Santos et al. 2019 reported the development of a method to detect transcripts during *R. prolixus* embryogenesis. Figure 5 shows the expression of several developmental genes at the early blastoderm (Stages 1 and 2) and during the segmented germ band stages (Stage 4 until Stage 7).

Another interesting topic for *R. prolixus* research regards the evolutionary mechanisms of germ cell formation. Two different mechanisms exist for germ cell specification: the separation via maternal germ plasm and zygotic induction from the mesoderm. The germ plasm is a specialized cytoplasm that accumulates at the posterior pole of the oocyte in *D. melanogaster* and another hymenopteran species *Nasonia vitripennis.* Germ plasm is inherited by the fertilized embryo and is essential for the formation of the pole cells early during embryogenesis. The pole cells remain dormant during embryogenesis and will form the gonads in the larval stages. Zygotic induction from the mesoderm is considered the ancestral stage among insects since it is present in the orthoptera *Gryllus bimaculatus* and in *O. fasciatus*, which is the milkweed bug (Ewen-Campen et al. 2011, 2013). Indeed, *oskar*, an essential factor for germ plasm formation in *D. melanogaster,* is not involved in this process in hemimetabolous species (Lynch and Roth 2011). Apparently, the molecular mechanisms that are involved in germ cell specification change during insect evolution, for example, *vasa* and *piwi* are not required for germ cell formation but only for gamete cell division in males (Ewen-Campen et al. 2013). Previous classical studies (Mellanby 1935; Heming and Huebner 1994) suggested that *R. prolixus* germ cells arise via the maternal germ plasm and that germ cells could be identified early in late blastoderm development. Recently, Brito et al. 2018 showed that *R. prolixus piwi* and *vasa* orthologues are specifically expressed in the germline during oogenesis. Knockdown of the *Rp-piwi* genes results in complete or partial sterility due to reduction in egg laying rates and embryo viability (Brito et al. 2018). These observations suggest that the molecular control of germline development might be partially different in *R. prolixus* and *O. fasciatus.* This recent study already points out the potential of the investigation of germ cell specification in *R. prolixus* and the possibility of the identification of novel genes with no obvious orthologues in *D. melanogaster.*

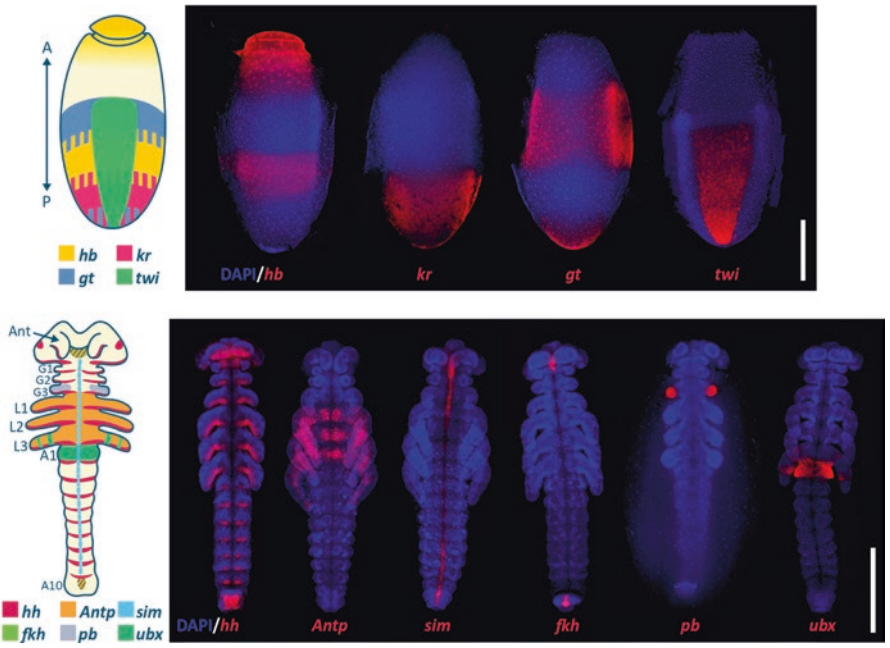

Fig. 5 In situ hybridization and fate map of *R. prolixus* at the blastodermal and segmented germ bands. Upper row—*R. prolixus* at uniform blastoderm (Stage 1) and embryos close to gastrulation process (Stage 3a) were stained for the nuclear marker DAPI and probed for developmental genes. Lower row—*R. prolixus* segmented germ bands (Stages between 6 and 8) were stained for the nuclear marker DAPI and probed for developmental genes. The schematic drawing summarizes the expression pattern at the early (Stages 1 and 2) and later segmented germ band stages. *hh- hedgehog*, *Antp- Antennapedia*, *sim—single-minded, Kr—Krüppel*, *Hb—Hünchback, gt—giant, pb-pairberry* and *twi—twist*

Gene expression profiles and mRNA localization during blastoderm stages exert a critical role in the subsequent morphogenetic stages of embryonic development. In the insect species that have been analysed so far, several genes are expressed in a localized pattern, establishing the AP and DV axes that drive the organized segmentation pattern and the formation of germ layers, respectively. However, an important distinction in embryogenesis modes is reflected in the expression patterns of these genes. The original knowledge about axial patterning in insects is derived from studies in *D. melanogaster*, which is the golden model of insect genetics. *D. melanogaster* is a holometabolous dipteran with fast embryonic development. It has a long-germ type of embryogenesis, where all segments are established almost simultaneously early in development and the embryonic region occupies almost the entire egg length (reviewed in Davis and Patel 2002; Roth 2004). Long germ embryos such as *D. melanogaster* and other derived Diptera also show an extreme reduction or absence of extra-embryonic membranes surrounding the embryo. In contrast, most basally branching insects show short-germ type embryogenesis, where a few anterior segments are generated early in one region of the embryo and the remaining

segments are formed via a secondary process of cell intercalation of the most posterior region of the embryos, which is also called the growth zone or segment addition zone (SAZ) (Fig. 2, McGregor et al. 2008, Auman and Chipman 2018). This latter type of segmentation via posterior patterning is considered the ancestral state among insects and even arthropods; thus, studies in hemipterans such as *R. prolixus* might help to unveil the ancestral control mechanism of segmentation in insects.

Expression analysis of four genes during the early stages *Krüppel (Kr)*, *Hünchback (Hb), giant (gt)* and *twist (twi)* provides interesting insights into *R. prolixus* AP and DV patterning (Fig. 5). *Rhodinus prolixus* embryos are short-germ type: (1) the germ rudiment occupies only half of the egg length; (2) only a few stripes of the segment polarity gene *hedgehog (hh)* are observed before posterior invagination and (3) the stripes of the segment polarity gene *hh* sequentially appear from the posterior region (see also Tobias-Santos et al. 2019). The short-germ type of embryogenesis is also typical of other hemipterans and several studies in the past years have investigated the role of segmental genes during *O. fasciatus* embryonic development (Liu and Kaufman 2004a, b; Angelini et al. 2005; Erezyilmaz et al. 2009a, b; Liu and Patel 2010; Birkan et al. 2011; Weisbrod et al. 2013; Stahi and Chipman 2016; Auman and Chipman 2018; Reding et al. 2019). A comparison of the *Hb* expression pattern in both hemipterans shows that the anterior domain of *Hb* expression in *R. prolixus (Rp-Hb)* is absent in *O. fasciatus*. This difference in *Hb* expression among both species can be explained either by an ancestral role of *Hb* in anterior patterning in hemipterans that was lost in *O. fasciatus* or that the *Rp-Hb* anterior domain of expression was acquired in *R. prolixus* and co-opted for anterior cap patterning.

A previous analysis of the roles of the other *gap* genes *Rp-kr* and *Rp-gt* during *R. prolixus* embryogenesis showed an essential role of both genes. While *Rp-gt* is required for head and abdomen formation, *Rp-kr* shows a classical *gap* phenotype (Lavore et al. 2012, 2014) that is consistent with the pattern of gene expression shown in Fig. 5. A recent analysis of another *gap* gene that was originally isolated from the beetle *T. castaneum* (Savard et al. 2006)*, mille-pattes (mlpt)* in *R. prolixus* provides evidence of a role of *Rp-mlpt* in the distinction between the thoracic and abdominal segments (Tobias-Santos et al. 2019). In the upcoming years, further analysis of the role of other *gap* genes will provide important insights into the evolution of the gene regulatory networks that are responsible for AP patterning in hemipterans. A global transcriptome approach isolated an anteriorly localized factor in the beetle *T. castaneum* that is an antagonist of the Wnt pathway (Schmitt-Engel et al. 2015; Ansari et al. 2018) and a cysteine-clamp gene that is responsible for embryonic polarity in the midge *Chironomus riparius* (Klomp et al. 2015). While it has not been possible to identify anteriorly or posteriorly localized maternally mRNAs in *O. fasciatus* by transcriptome analysis (Ginzburg et al. 2017), similar future studies in *R. prolixus* could unveil new maternally localized factors in the kissing bug eggs since these eggs do display a clear axial distinction when they are laid.

The expression of the dorsoventral gene *twist (twi)* in *R. prolixus* embryogenesis at the ventral side (Fig. 5) marks the prospective mesoderm that will invaginate at

later stages. The gene *twi* is a BHLH transcription factor and is essential for mesoderm specification in several arthropods (reviewed in Moussian and Roth 2005). *Rp-twi* expression has been shown by RT-PCR to be regulated by the dorsal/toll pathway in *R. prolixus* (Berni et al. 2014), and this is the major pathway required for DV patterning in all insect species that has been investigated so far (Nunes da Fonseca et al. 2008; Sachs et al. 2015). Since the formation of the DV axis in *O. fasciatus* DV has been thoroughly investigated and a dynamic BMP pathway polarized by the toll pathway was described (Sachs et al. 2015), it will also be important to determine if the same hierarchies among the toll and BMP pathways are evident in *R. prolixus* DV patterning.

Another under investigated process in insects concerns the process of maternal-zygotic transition (MZT). All of the metazoan species that have been investigated so far undergo MZT, which consists of genome-wide maternal mRNA degradation followed by zygotic transcription. In *D. melanogaster,* MZT takes place during the first 2 h of embryonic development, and zygotic activation is largely controlled by the zinc-finger transcription factor *zelda (zld).* Functional analysis via pRNAi knockdown of the *zld* orthologue in *R. prolixus* led to problems in cellularization during the first 12 h of development, in addition to defects in embryonic posterior segmentation at later stages (Ribeiro et al. 2017). Previous comparisons by RT-PCR of gene expression in non-fertilized and fertilized *R. prolixus* eggs were able to detect differences in expression at only 6 h AEL (Berni et al. 2014), suggesting that maternal mRNA degradation and zygotic transcription might start very early during embryogenesis. The future comparison of the transcriptomes of non-fertilized and fertilized eggs could provide information about the timing of global activation of zygotic transcription in *R. prolixus*.

The analysis of the expression of developmental markers during segmented germ band stages also provides important insights into *R. prolixus* embryogenesis (Fig. 5). One of the most conserved markers of central nervous system ventral midline cells in several arthropods (Linne et al. 2012), *single-minded (sim),* is also expressed in the ventral midline of *R. prolixus* (Fig. 5). Stomodeum and proctodeum formation are marked by the expression of the transcription factor *forkhead (fkh)*. Both ectodermal structures also express *fkh* in *R. prolixus*, suggesting that the ectodermal component of gut formation shows at least one conserved regulatory aspect (Fig. 5). It will be interesting to investigate the role of the orthologues of endodermal genes presumably acting in *R. prolixus* gut patterning such as the transcription factors *Pt-serpent (Pt-srp) and Pt-hepatocyte-nuclear factor-4 (Pt-hnf-4)*, which have both been shown to be involved in endoderm formation in other arthropod groups (Murakami et al. 2005; Feitosa et al. 2017).

Hox genes are transcription factors that are responsible for providing the identity of segments in bilaterian animals (Akam 1995). In *R. prolixus* Hox gene expression of *Antp* is observed, as expected, in the thoracic region, *Ubx* in the first abdominal segment and in the posterior legs, and *Pairberry (Pb)* in the labial segment (Fig. 5). These expression domains are conserved among arthropods and other Hemiptera (Angelini et al. 2005). Lastly, the *hh* gene is expressed in the posterior region of every segment and in the ectodermal part of the gut, the stomodeum and the

proctodeum (Fig. 5). All of these expression domains are present in *R. prolixus*, corroborating the hypothesis that the patterning of the ectodermal part of the gut is conserved.

5 Future Directions of *R. prolixus* Embryogenesis Research

As presented in the present chapter, *R. prolixus* can presently be considered a great species to investigate the embryonic development of Hemiptera, which is the largest hemimetabolan insect order by number of species. In addition, the availability of the *R. prolixus* genome (Mesquita et al. 2015) and the transcriptomes of several developmental stages and tissues (Medeiros et al. 2011; Ribeiro et al. 2014; Lavore et al. 2015; Brito et al. 2018) enable *in silico* comparative expression using data available in public databases. In the past, several techniques have been established for the investigation of gene function. Changes and adaptations in methods that have been adapted from other insects have allowed fixative agents to overcome the hard and previously believed impenetrable eggshell of *R. prolixus.* Thus far, only single-colour in situ hybridization studies were established and the development of two- or three-colour in situ hybridization should be established in future studies to allow further detailed comparisons.

Another technological advance that should greatly impact *R. prolixus* biology is the development of genome editing tools. Genome editing by CRISPR/Cas9 has been used to generate knockout and transgenic lines in several insect species (Bassett et al. 2014; Gilles et al. 2015; Cardoso et al. 2017; Chaverra-Rodriguez et al. 2018). For *R. prolixus*, the development of such tools requires the identification of proper drivers for germline expression and methodologies aimed at delivering DNA into the embryo, which are currently underway (Berni et al. unpublished). These should allow the generation of gene knockdowns and fluorescent-tagged reporter and overexpression transgenic lineages, thus advancing the possibilities of investigation by the whole scientific community.

An important topic of research for upcoming years in *R. prolixus* will be the evolution of the molecular control of AP and DV axis formation. Investigations over recent years have established that conserved genes do play a role in AP and DV axis formation, but extensive variation and the introduction of new genes and changes in the molecular pathways have occurred. In general, the zygotic components that are responsible for AP and DV axis formation have been conserved in insect evolution, while some maternal components have been introduced into these complex gene regulatory networks (Lynch and Roth 2011; Klomp et al. 2015; Ansari et al. 2018).

Studies on *R. prolixus* embryogenesis provide a unique opportunity to connect the vast knowledge on the physiology, biochemistry and bioenergetics of an important vector species with the knowledge of the signalling pathways that have been studied in traditional and genetic model systems such as the fruit fly *D. melanogaster* and the beetle *T. castaneum*.

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Anatomy of the Nervous System of Triatomines

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Abstract The nervous system of triatomines follows the general plan for insects in terms of organization. Yet, its anatomy is in line with the form of the different regions of the bug's body. The brain is located in the posterior region of the elongated head, the cavity of which is mostly occupied by the cibarial pump. The ganglia composing the ventral nerve cord exhibit a high degree of fusion, conforming a mass of suboesophageal or gnathal ganglia, a prothoracic ganglion and a unique posterior nervous mass, formed by the fusion of meso- and meta-thoracic and segmental abdominal ganglia. Most of the integration centres (i.e. neuropils) of the brain and ganglia are less structured than in other insects, but they can be easily recognized by histological inspection. In the brain, all major centres are well defined, and in the ganglia it is possible to establish the correspondence of each neuropil with its respective thoracic or abdominal segment. We have tried to keep the nomenclature employed for naming different structures as clear as possible. In some cases, synonymy is indicated, in order to allow the reader to find equivalents in the literature concerning other insects, which could have adopted different criteria. This chapter presents a synthesis of our present knowledge of the neuroanatomy of kissing bugs, underlining its particularities. A general description is presented here, but much work is still needed to elaborate an atlas as detailed as those existing for domestic and fruit flies or honey bees.

Keywords Neuroanatomy · Brain · Central nervous system · Ventral nerve cord · Ganglia · Nerves

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1 Introduction

The insect nervous system can be divided in three morpho-functionally distinct portions: (1) the central nervous system, (2) the Peripheral Nervous System and (3) the stomodeal nervous system.

The central nervous system is constituted by the brain, lying above the anterior end of the stomodaeum and the ventral cord, composed of median segmental ganglia and paired connectives, lying beneath the alimentary canal. The two parts are joined by connectives embracing the stomodaeum. In the ganglia, cell somata are restricted to the peripheral region, while the central area is occupied by neuropils formed by nervous fibres (axons and dendrites) and their synapses. The insect brain also features abundant bundles of neuronal fibres connecting different neuropils. Fascicles and tracts connect two different brain regions ipsilaterally and commissures connect two regions contralaterally. The form of the brain is variable in different insects, but it always shows a differentiation into three parts, the protocerebrum, the deutocerebrum and the tritocerebrum, originated by the fusion of anterior nervous masses. Figure 1 depicts a diagrammatic representation of the basic anatomy of the insect brain (not to scale).

The protocerebrum is the dorsal and largest part of the brain, hosting superior integration centres and visual neuropils. It includes the dorso-lateral protocerebral

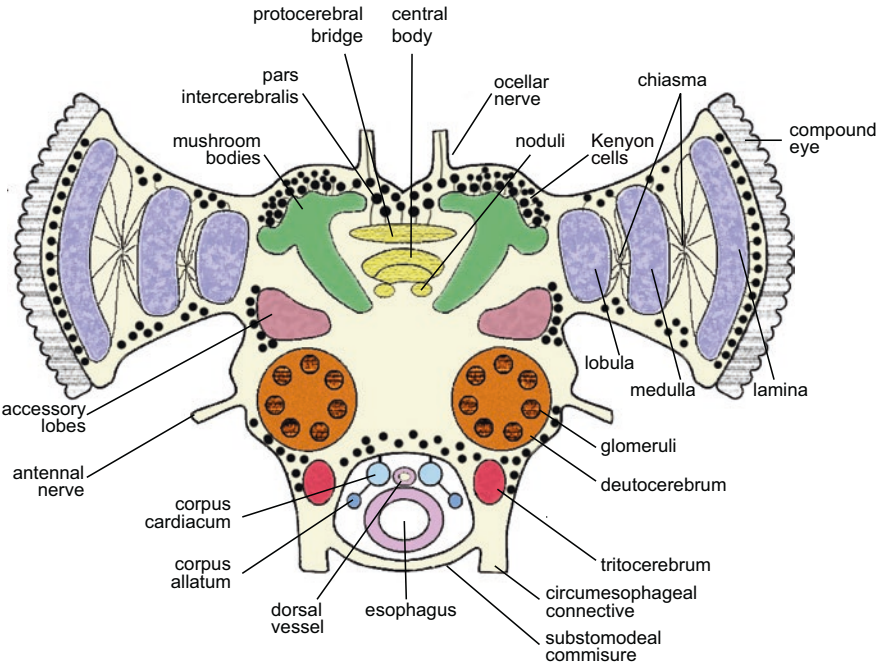

Fig. 1 Diagrammatic representation of the basic anatomy of the insect brain showing the main regions of organized neuropils. The black dots indicate the location of cells bodies. (Not to scale)

lobes, situated on either side of the pars intercerebralis connecting both sides of the brain and sometimes, lateral accessory lobes. The cells of the pars intercerebralis, a group of somata on either side of the midline, contribute to form the ocellar nerves and the protocerebral bridge (pons cerebralis), a median mass of neuropil connecting with many other parts of the brain. Also, within the pars intercerebralis are located neurosecretory cells, whose axons extend to the corpora cardiaca (neurohemal organ for several different hormones). Besides the protocerebral lobes, the other remarkable neuropils of this region are a pair of mushroom bodies (corpora pedunculata) and a median central complex.

The mushroom bodies (corpora pedunculata) are conspicuous paired neuropils in the brain of insects, located at the sides of the pars intercerebralis (medial protocerebrum). They are given their shape by the processes of a large number of morphologically similar interneurons, called Kenyon cells (Dujardin 1850; Kenyon 1896; Mobbs 1985), located in the dorsoposterior protocerebrum. The Kenyon cells extend their axon-like processes to build the calyx, the distal neuropil of the mushroom body, then run through a pedunculus and divide to form a system of lobes. At the pedunculus and the lobes, the Kenyon cells axons make synaptic connections with dendrites of extrinsic (output) neurons (Hanström 1940; Strausfeld et al. 1995; Fharbach 2006). The mushroom bodies are considered as multimodal integration centres, best studied for their role in learning and memory. These structures have been largely analysed in social hymenopterans, particularly the honey bee *Apis mellifera*. In the cockroach *Periplaneta americana* and the fruit fly *Drosophila melanogaster* they have also been the subject of numerous studies (reviewed in: Heisenberg 1998; Strausfeld et al. 1998; Strausfeld 2012).

The central complex is an ensemble of interconnected neuropils, which span the midline of the protocerebrum. From rostral to caudal these neuropiles include the protocerebral bridge, the central body consisting in the upper division (fan-shaped body) and the lower division (ellipsoid body) and a pair of noduli at the base. The central complex is associated with other satellite neuropils, the largest of which are the lateral accessory lobes, also referred to as the ventral bodies. In the unified nomenclature proposed by Ito et al. (2014), all this region is also called ‘Lateral Complex’.

The lobes of the protocerebrum bear laterally the large optic lobes, which contain the visual centres of the compound eyes. Each consist of three successive neuropils known, from distal to proximal, as the lamina, the medulla and the lobula complex, which is subdivided into the lobula and lobula plate in the Lepidoptera, Trichoptera and Diptera. Between two successive neuropils, the fibres cross over horizontally, forming the outer and inner optic chiasmata. In the lamina of most insects, the axons of retinula cells from one ommatidium remain together and are associated with neurons originating in the lamina and medulla to form a cartridge. Axons from most of the retinula cells in the eye end in the lamina cartridges, although one or two from each ommatidium pass through to the medulla. The ocellar centres are located in the distal part of the ocellar pedicels, which connect the ocelli with the brain. The somata of these neurons are situated antero-dorsally in the region of the pars intercerebralis.

The deutocerebrum is the second section of the brain, receiving inputs from the antennal nerves. It comprises the paired antennal (olfactory) lobes and antennal mechanosensory and motor centre.

The antennal mechanosensory and motor centre contains the terminal arborizations of mechanosensory neurons from the antennal scapus and pedicel and possibly also from the flagellum. It also contains dendritic arborizations of the motor neurons controlling the antennal muscles.

The sensory fibres of the antennal nerve trunks terminate in numerous glomeruli distributed in the periphery of the antennal lobes; each sensory axon going to a single glomerulus in most insects. Within a species, individual glomeruli appear to be constant in form and position. The antennal glomeruli of opposite sides are connected by a fibrous deutocerebral commissure, which runs through the lower part of the brain.

The tritocerebrum, the third part of the brain, is the smallest one and consists of a pair of lobes beneath the deutocerebrum. From it, the circumesophageal connectives pass to the suboesophageal ganglion (gnathal ganglia). The tritocerebral lobes of either side are connected by a substomodeal commissure passing behind the oesophagus. The principal nerves of the tritocerebrum in insects are the frontal ganglion connectives and the labral nerves.

The ventral nerve cord comprises the post-oral series of segmental ganglia and their connectives. Morphologically, the ventral nerve cord begins with the three gnathal ganglia of the head forming the suboesophageal ganglion and includes as well the ganglia of the thorax and abdomen. The gnathal ganglia are always united to each other in the mature insects to form the second composite nerve mass of the head known as the suboesophageal ganglion.

The thoracic region of the insect body comprises three median ganglia corresponding to the three thoracic segments, namely prothoracic ganglion, mesothoracic ganglion and metathoracic ganglion. Frequently the mesothoracic and metathoracic ganglia are united, and the definitive ganglion of the metathorax may include one or more abdominal ganglia. In the insect abdomen, there are, at the most, eight definitive segmental ganglia corresponding to the first eight segmental somites, but the last is always a composite ganglion, which innervates the eighth and following segments. Frequently, one or more of the posterior ganglia are combined, but the nerves from each ganglion always go to the segment in which the ganglion had its origin. The ganglia of the ventral nerve cord tend to fuse in diverse combinations in different insects.

The peripheral nervous system is constituted by the axons of the motor neurons innervating peripheral structures (muscles and glands), the cell bodies of which are contained in the central ganglia and of the axons, cell bodies and terminal processes of sensory neurons, which are always bipolar or multipolar.

The stomodeal nervous system is composed by the frontal ganglion as the more constant element, in addition to the paired hypocerebral ganglia and, sometimes, an ingluvial ganglion. The stomodeal nervous system of insects has a dual function, as the sympathetic innervation of organs and as a site of endocrine activity. The specialized secretory portions of this system are a part of a complex incretory unit known as the intercerebralis-cardiacum-allatum system.

Additional information about the general structure of the insect nervous system can be found in works by Snodgrass (1935), Pflugfelder (1937), Homberg (2008), Strausfeld (2012) and Chapman (2013) and the work by Ito et al. (2014) presents a consensual proposition of unified nomenclature.

The central nervous system of Heteroptera presents a complete fusion of the abdominal ganglia. In most land-bugs (e.g. *Rhodnius* spp.), the suboesophageal and prothoracic ganglia remain independent, whereas the mesothoracic, metathoracic and abdominal ganglia are fused into a unique nervous mass. The abdomen is innervated by five pairs of segmental nerves, the median pair being the longest and divided posteriorly in the genital segments. The stomodeal nervous system comprises the frontal ganglion and commissures, a single recurrent nerve and a median hypocerebral ganglion. In addition, paired or fused corpora cardiaca and corpora allata are closely associated with the wall of the dorsal aorta (Horridge 1965).

Among Triatomines, *Rhodnius prolixus* and *Triatoma infestans* are the most studied species and a considerable body of information has been published on different aspects of their physiology and behaviour. Wigglesworth (1954) based his classical studies on the endocrine regulation of moult and metamorphosis on *R. prolixus*, which then became a classical model in insect physiology. In the past few years, considerable attention has been focused on different aspects of their sensory physiology and behaviour (e.g. Guerenstein and Lazzari 2009; Lazzari et al. 2013; Barrozo et al. 2017). Yet, our knowledge about the neuroanatomy of triatomines remains fragmentary, provided that this aspect has been less studied than other facets of their biology. A list of the principal contributions is as follows:

Wigglesworth (1959) described the histological organization of the prothoracic and terminal ganglia of *R. prolixus,* Barth (1952, 1975) performed studies on the central ganglia of *T. infestans* and Insausti (1994) has described the anatomy of the nervous system of this species in detail. Additional studies on particular aspects of the nervous system of *R. prolixus* and *T. infestans* have been published by Maddrell (1963, 1966), Pinet (1963), Ramirez Perez (1969), Anwyl (1972), Anwyl and Finlayson (1974), Morris and Steel (1975), Faruqui (1977), Flanagan (1984, 1986), Insausti and Lazzari (1996, 2000, 2002), Settembrini (1999, 2003, 2004, 2008, 2005, 2011), Barrozo et al. (2009). Vafopoulou and collaborators (2019) recently described the localization of neuropeptide- and serotonin-producing cells in the brain of *R. prolixus*, which are associated with the circadian clock.

2 The Nervous System of Triatomines

2.1 General Morphology

The head of triatomines has a mostly tubular shape, which shrinks slightly at the basis of the antennae and on its posterior part, just before the thorax. The sucking apparatus, with the large cibarial pump musculature, occupies its anterior portion;

as a consequence, the brain is located in the occipital region of the cephalic capsule, widely fused to the suboesophageal ganglion (Fig. 2).

As in other insects, the most voluminous portion of the triatomine brain corresponds to the bilobed protocerebrum, which covers dorsally the rest of the brain and from which the optic lobes project laterally. They are externally separated from the central lobes of the protocerebrum by a slightly pronounced constriction. Two ocellar nerves emerge from the central area of each protocerebral lobe. In the posterior region of the latter, two thin nerves related to the corpora cardiaca and a third tegumentary nerve originate (Fig. 3).

The deutocerebrum is much smaller than the protocerebrum and occupies the ventrolateral region of the brain. Externally, it appears as two little pronounced lobes, from which thick antennal nerves and thin tegumentary nerves emerge (Figs. 3 and 4).

The tritocerebrum is the smallest portion of the triatomine brain and is located medially to the deutocerebrum, associated with the oesophagus by its internal side. The tritocerebral commissure can only be revealed by histology (Fig. 13). Although non-evident externally, the division between deuto- and trito-cerebrum can be estimated by the origin of the frontolabral nerves. These short, thick nerves

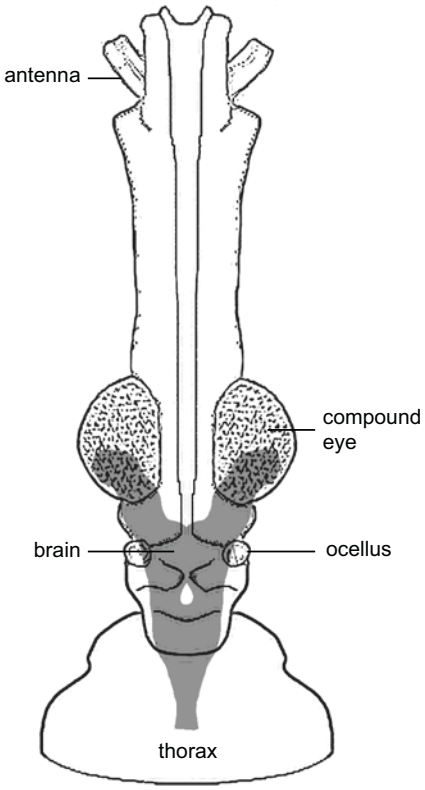

Fig. 2 Diagrammatic representation of the head of *Rhodnius prolixus* showing the position of the brain

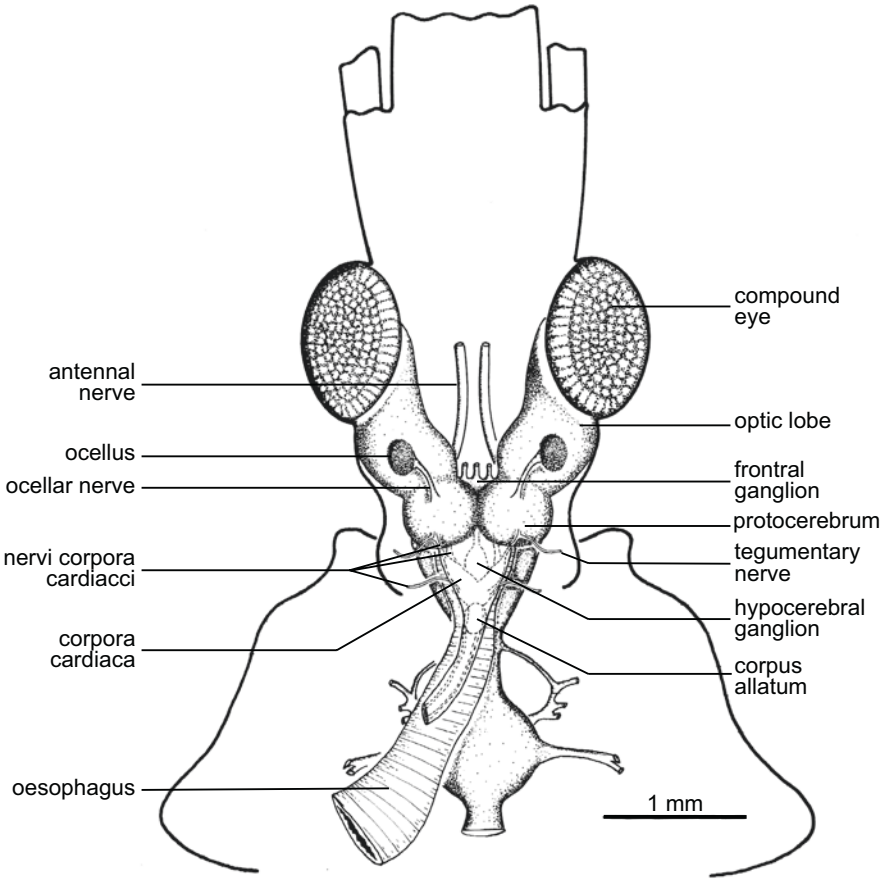

Fig. 3 Diagram of the dorsal view of the brain, suboesophageal ganglion, frontal ganglion and prothoracic ganglion of *Triatoma infestans*. The retrocerebral complex and the hypocerebral ganglion, placed below the aorta are shown using dotted lines

immediately divide into the short, medially directed frontal nerves and the long, slender, anteriorly directed labral nerves. The labral nerves run ventrally to the clypeus-labral region of the head. The frontal nerves connect with the frontal ganglion over the midline of the oesophagus. From the anterior portion of the frontal ganglion, a median frontal nerve arises and runs anteriorly, innervating the voluminous cibarial dilatory muscles. The ganglion narrows posteriorly into a recurrent nerve that runs between the brain and the suboesophageal ganglion, dorsal to the oesophagus and continues below the origin of the aorta, where it joins the unpaired hypocerebral ganglion (Fig. 3). The frontal ganglion is, therefore, the connection between the central and the stomatogastric nervous system and itself the centre of the latter.

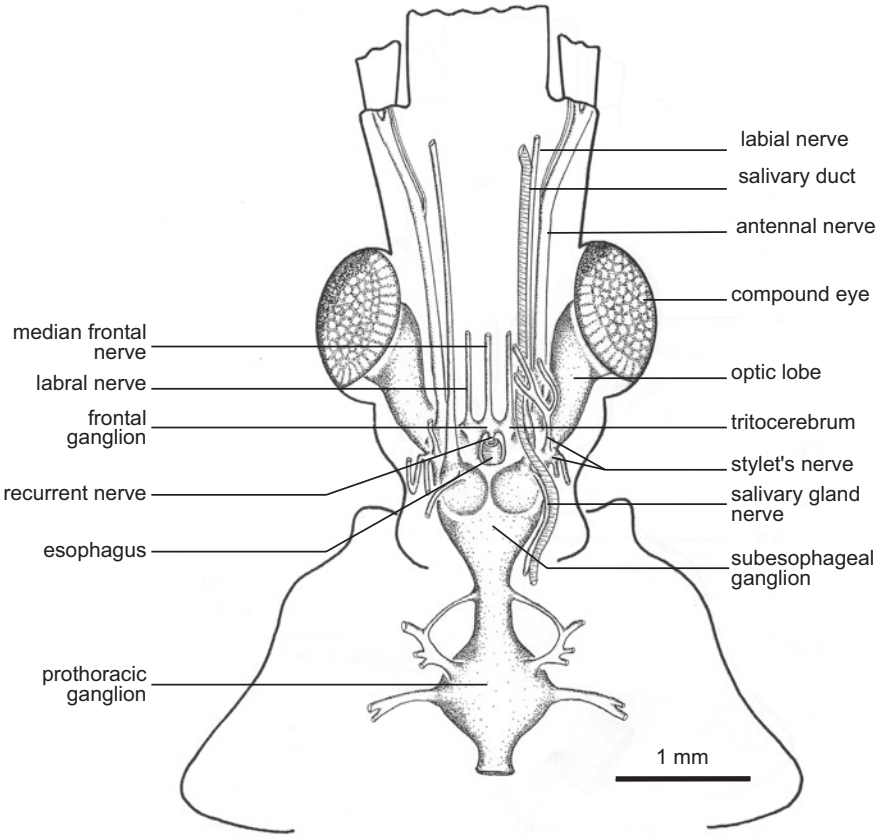

Fig. 4 Diagram of the ventral view of the brain, frontal ganglion, suboesophageal ganglion and prothoracic ganglion of *Triatoma infestans.* The origin of the main nerves is shown

The boundary between the brain and the gnathal ganglia (suboesophageal ganglion) is marked externally by the protocerebral lobes dorsally and the deutocerebral lobes ventrally. The circumoesophageal connectives are apparent only histologically. From the anterolateral region of this ganglionic mass, just below the boundary with the brain, the nerves controlling the muscles of the stylets originate and a slender nerve, which goes to the corpora cardiaca. A pair of thick, conspicuous labial nerves arises from the ventral surface of the suboesophageal ganglion lobes and runs ventrally into the labium. These nerves run very close together with the salivary duct. A very slender nerve arises ventrally from the base of the labial nerve and runs posteriorly to join the salivary duct, innervating the salivary glands in the thorax (Fig. 4).

The suboesophageal ganglion joins the prothoracic ganglion by means of longitudinal connectives fused medially. The prothoracic ganglion is located in the prosternum, below a pair of apodemes. This ganglion provides innervation to the

prothorax. The meso- and meta-thoracic ganglia are fused to the abdominal ones, giving place to a unique nervous mass, the posterior ganglion, located in the mesosternum (Fig. 5). From this mass all the nerves that assist the thorax and the abdomen originate. The abdomen is supplied by five pairs of nerves, the first four pairs are relatively fine and innervate the first four segments of the abdomen. The fifth and following abdominal segments are supplied by branches of the stout median abdominal nerve five. This nerve also innervates the reproductive organs and the rectal sac (Fig. 6).

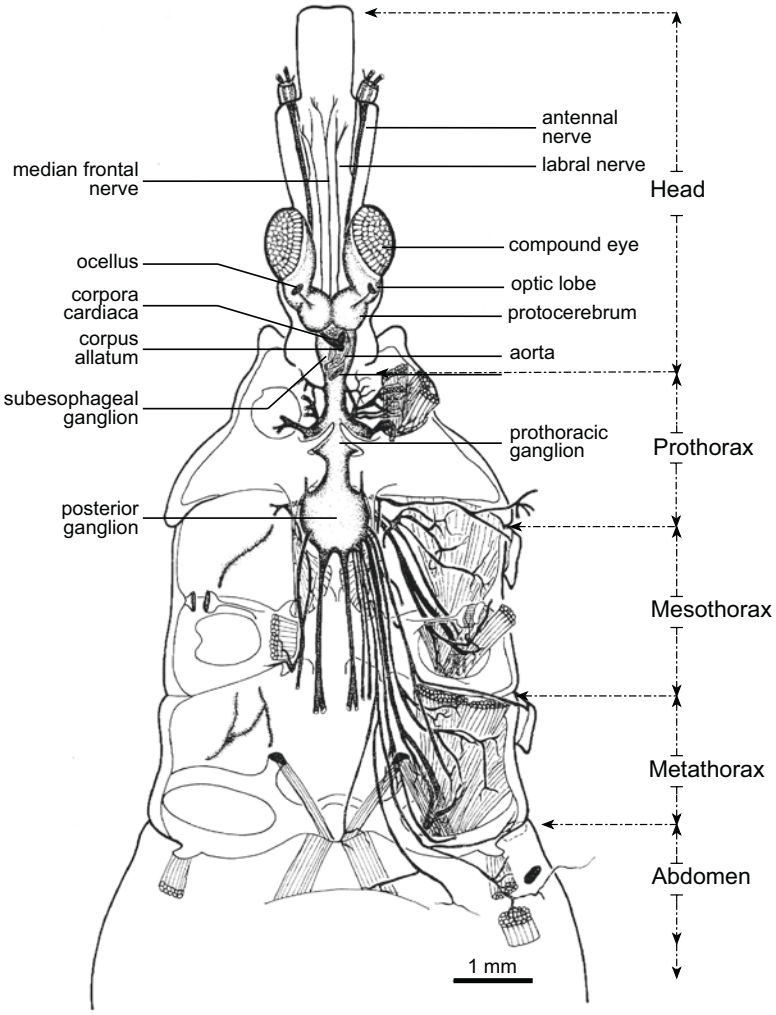

Fig. 5 Diagram of the dorsal view of the central nervous system and peripheral nerves of *Triatoma infestans*. Ventral and leg muscles and their innervation are shown

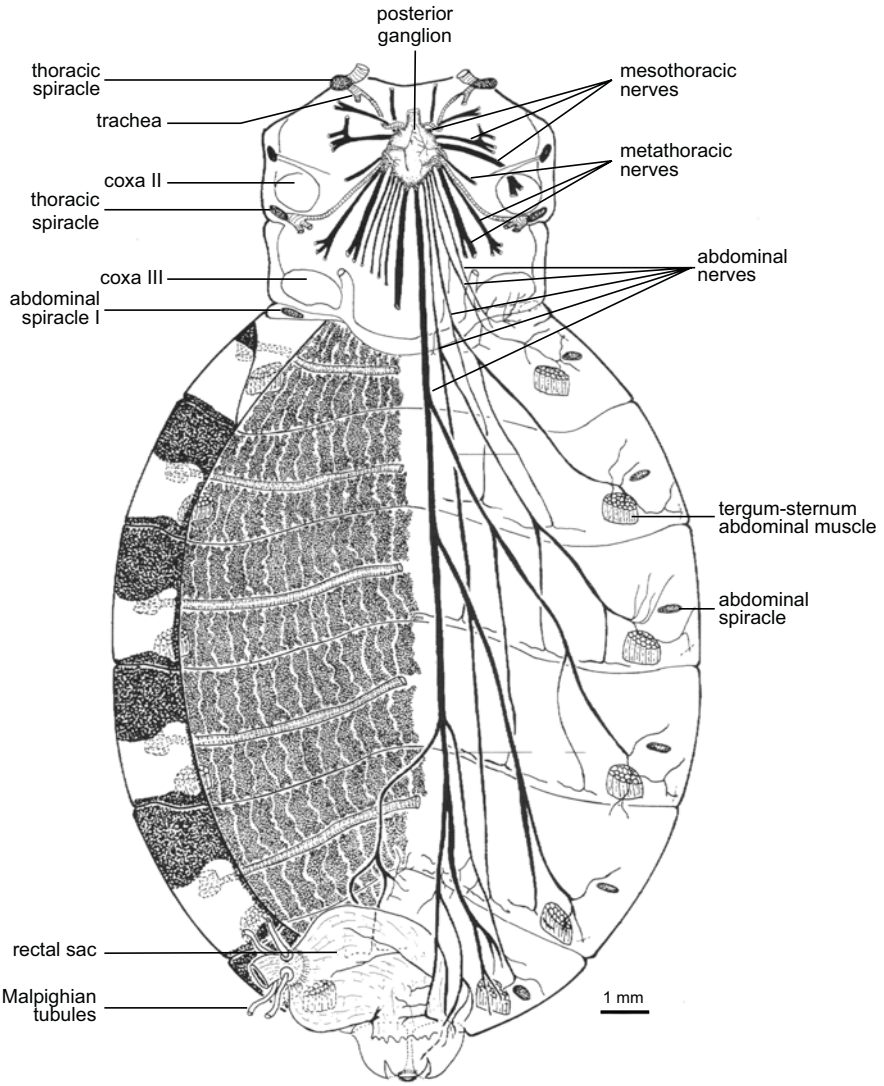

Fig. 6 Diagram of the dorsal view of the posterior fused ganglion (mesothoracic + metathoracic + abdominal ganglia). The innervation of the abdominal segments and rectum is shown

2.2 The Brain

Protocerebrum

The **protocerebral lobes** in *T. infestans* and *R. prolixus* consist of two large globular neuropil masses, which are situated on either side of the median pars intercerebralis and the lateral accessory lobes. The protocerebral lobes cover most of the dorsal side of the brain. Both lobes are connected with each other by a protocerebral commissure (Fig. 7).

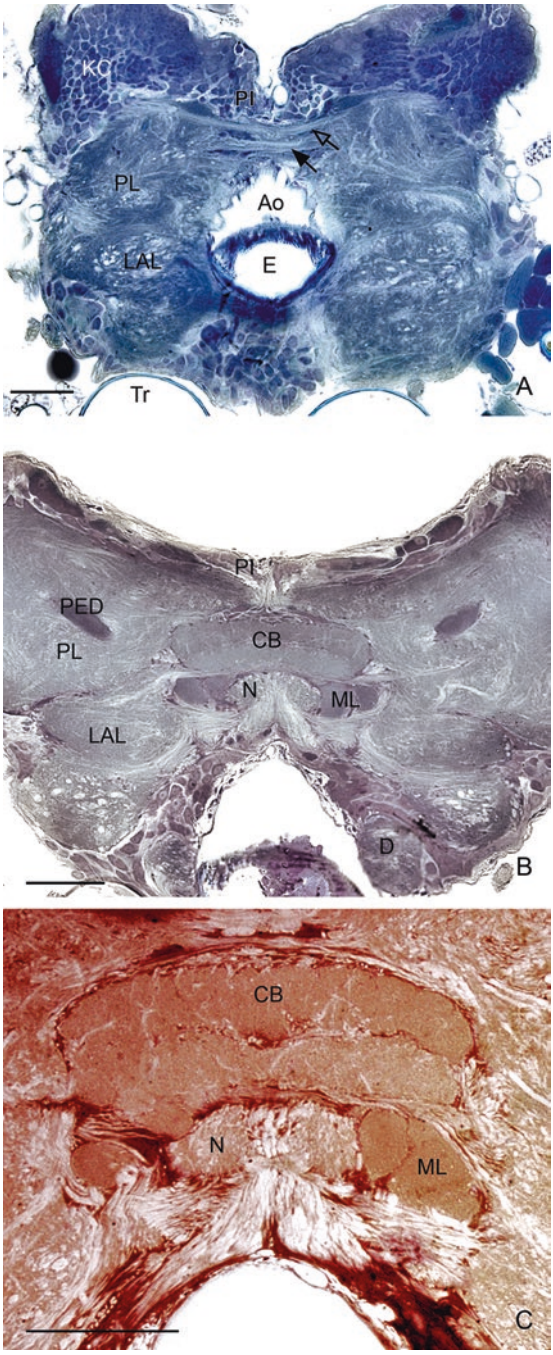

Fig. 7 Light micrographs of frontal sections of the brain of *Rhodnius prolixus* (**a**) Section across the anterior protocerebrum showing the protocerebral lobes. The filled arrow indicates the protocerebral bridge, the empty arrow indicates the protocerebral commissure. Scale bar 50 µm
(**b**) Section across the protocerebrum at the level of the central body. Scale bar 50 µm
(**c**) Detail of the central body. Scale bar 50 µm
Ao aorta, *CB* central body, *E* oesophagus, *KC* Kenyon cells, *LAL* lateral accessory lobes, *ML* medial lobes of the mushroom bodies, *N* noduli, *PED* peduncle of the mushroom bodies, *PI* pars intercerebralis, *PL* protocerebral lobes, *Tr* trachea

The **central complex** is well-defined and placed anteriorly in the central part of the protocerebrum. It comprises the protocerebral bridge dorsal to the central body, which consists of an upper division and a lower division and the noduli at its base (Fig. 7).

The **mushroom bodies** of *T. infestans* and *R. prolixus* are noticeably different in their morphological appearance from the mushroom bodies of honey bees and cockroaches. In these triatomine species the Kenyon cells have small somata located antero-dorsally in the protocerebral lobes and their axon-like processes supply a single calyx. The calyx is roughly globular, surrounded by the Kenyon cells. The stalk (peduncle) runs ventrally and slightly anteriorly before dividing into a system of two main lobes, the vertical and medial lobes. The medial lobe is divided into three bulbous subdivisions and extends posteriorly next to the antennal lobe (Figs. 8 and 9).

The voluminous **optic lobes** comprise three well-developed neuropil masses, namely the lamina, the medulla and the lobula, surrounded by ganglion cells (Fig. 10). The lamina, the outermost neuropil mass beneath the ommatidial layer, is a slightly arched, flattened structure in which the retinal axons form fibrous bundles (probably cartridges) (Fig. 10b). The various fibres cross each other so as to form the outer chiasma between the lamina and the medulla (Figs. 10a, b). The medulla is the most voluminous neuropil of the optic lobe, globular in shape and organized in two divisions (Fig. 10c). The lobula is oval in shape and presents three distinguishable lobes (Fig. 11b).

Triatomine bugs possess two well-developed **ocelli**, located in a latero-dorsal position, behind the compound eyes (Fig. 2). The ocelli of *T. infestans* exhibit an unusual degree of complexity, as compared with other insects. The analysis of the ocellar pathways revealed direct connections by first-order interneurons with nervous centres located in the brain and several neuromeres along the ventral nerve cord. The axons of the photoreceptors synapse with second-order neurons in the peripheral neuropil at the base of the ocelli. Each neuropil is connected to the brain by an ocellar nerve (Figs. 8a and 12a). The fibres of the ocellar nerves continue inside the brain as lateral tracts, through the superficial dorsal protocerebrum. They run between the calyces of the mushroom bodies and dorsal to the central body, connecting to different regions of the brain, to the gnathal ganglia and to the thoracic ganglia. Cell bodies are located in the dorsal protocerebral lobes, in the pars intercerebralis and the ipsilateral posterior protocerebrum (Insausti and Lazzari 1996, 2002).

Fig. 8 continued (**c**) Sagittal section of the brain of *Triatoma infestans* showing the orientation of the mushroom body (dotted line), the asterisk indicates the calyx, the empty arrow indicates the pedunculus and the filled arrow indicates the medial lobes. Scale bar 100 μm
(**d**) Detail of the mushroom body and central body of *Rhodnius prolixus* in frontal section. The bundle of fibres that relates both structures is evinced. Scale bar 50 μm
Ao aorta, *E* oesophagus, *CB* central body, *D* deutocerebrum, *KC* Kenyon cells, *LAL* lateral accessory lobes, *MBCA* mushroom body calyx, *O* ocellus, *ON* ocellar nerve, *PI* pars intercerebralis, *PL* protocerebral lobes, *Tr* trachea

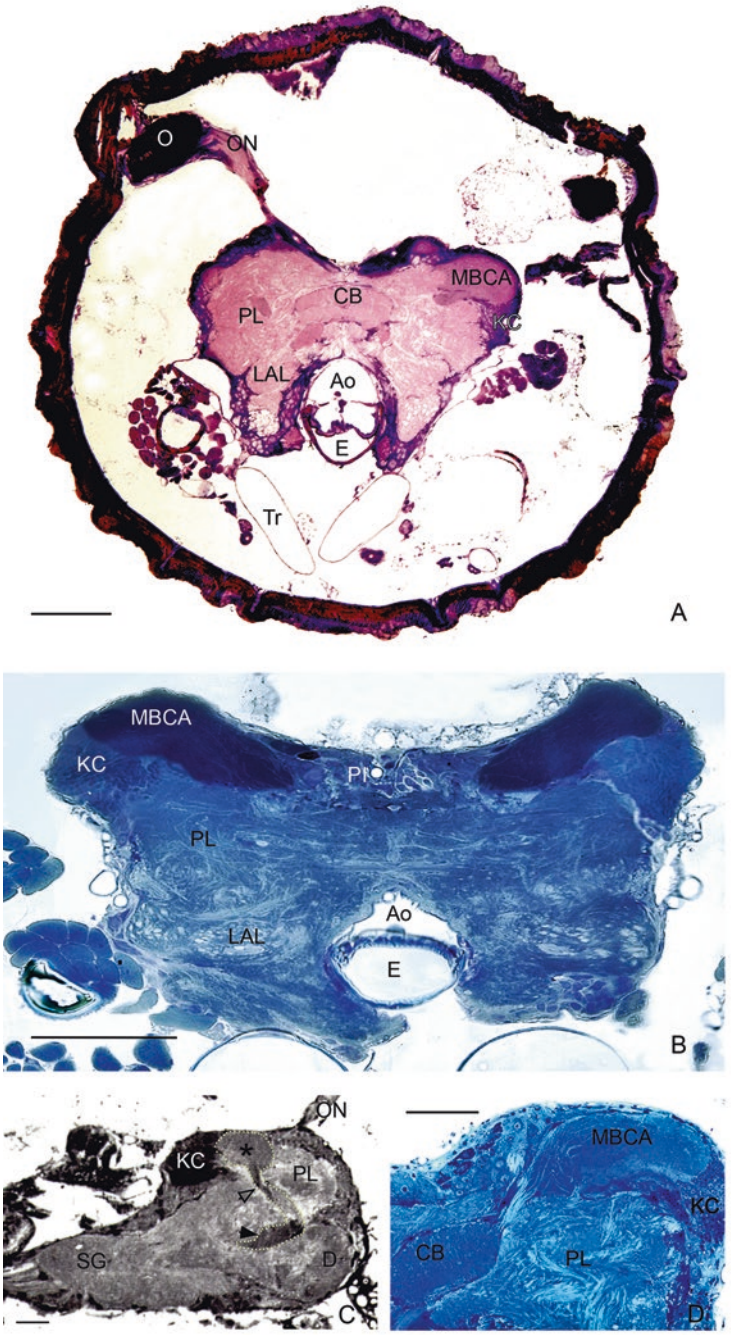

Fig. 8 (**a**) Light micrograph of a frontal section of the head of *Rhodnius prolixus*. Scale bar 100 μm (**b**) Frontal section of the protocerebrum of *Rhodnius prolixus*, showing the mushroom bodies, calices and Kenyon cells. Scale bar 50 μm

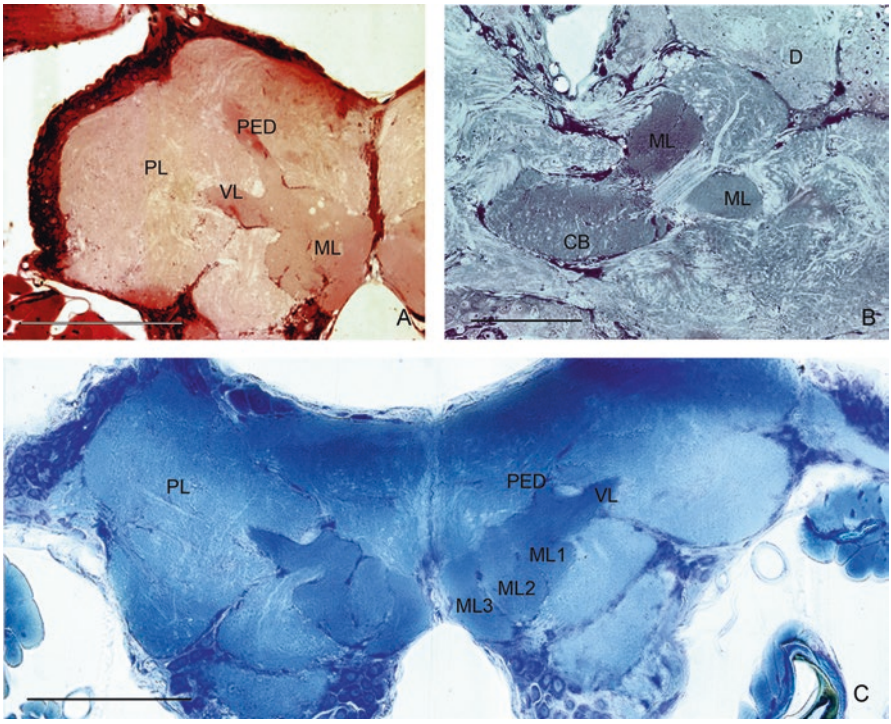

Fig. 9 Light micrographs of the brain of *Rhodnius prolixus*
(**a**) Frontal section at the level of the lobes of the mushroom body. Scale bar 50 μm
(**b**) Detail of a horizontal section of the brain, showing the bundle of fibres relating the central body to the deutocerebrum and the medial lobes of the mushroom bodies. Scale bar 50 μm
(**c**) Frontal section showing the lobes of the mushroom bodies. Scale bar 50 μm
CB central body, *D* deutocerebrum, *ML* 1-2-3, medial lobes of the mushroom body, *VL* vertical lobe of the mushroom body, *PED* peduncle of the mushroom body, *PL* protocerebral lobes. Scale bar 50 μm

Deutocerebrum

The deutocerebrum is the second largest part of the triatomine brain, next to the protocerebrum. It consists of a pair of large neuropil masses, each one organized in two parts: the antennal lobe (AL) and the antennal motor and mechanosensory centre (AMMC), also called dorsal lobe in some insects. Both neuropils receive sensory afferents originating from the antenna (Figs. 11 and 12).

The antennal lobe is ellipsoid in shape, flattened in the dorso-ventral axis. It is organized, as in other insects, in glomerular units surrounding a central fibre core (Fig. 12). Barrozo et al. (2009) established a partial three-dimensional map of the antennal lobe of *R. prolixus*, the first in a hemipteran insect. The antennal lobes of this species are relatively diffuse structures, which nevertheless show a glomerular

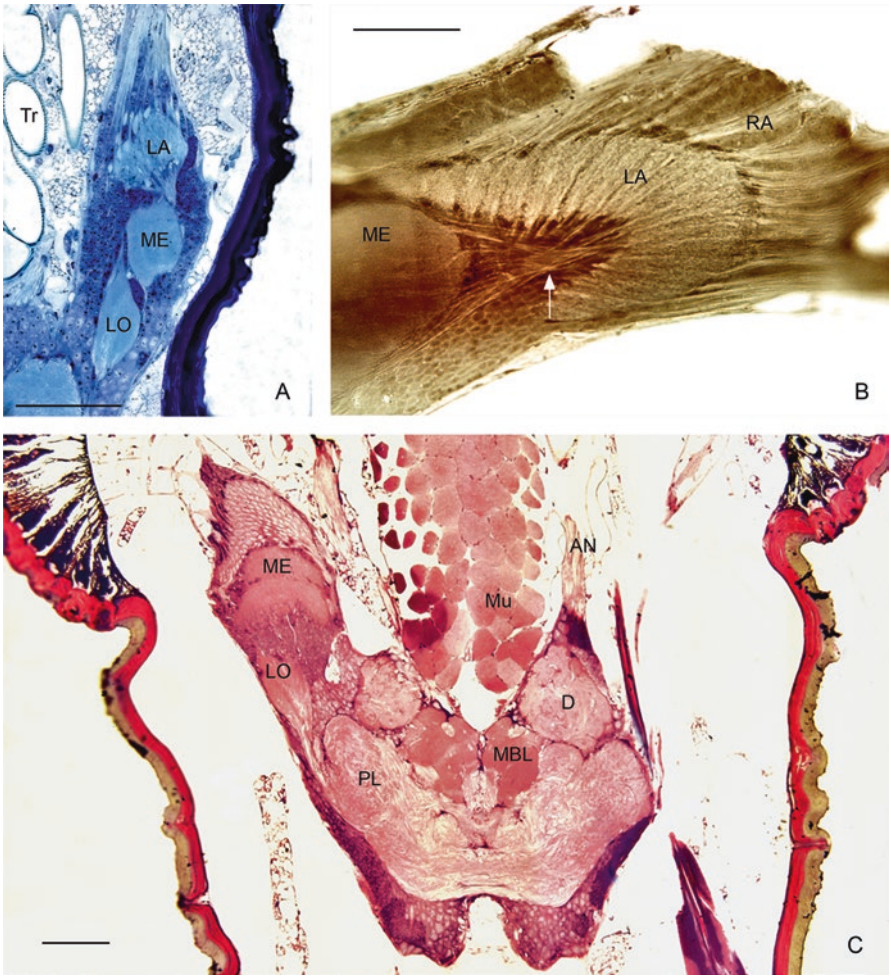

Fig. 10 Light micrographs of horizontal sections of the brain of *Rhodnius prolixus*
(**a**) Section of the optic lobe showing the three neuropilar masses, lamina, medulla and lobula. Scale bar 50 μm
(**b**) Detail of the lamina neuropil of the optic lobe. The retinal axons form fibrous bundles (cartridges?). The various fibres cross each other to form the outer chiasma (arrow)
(**c**) General view of the brain. The left side of the brain shows the optic lobe with the divided medulla and lobula. The right side shows the antennal lobe (deutocerebrum) and the antennal nerve. Scale bar 100 μm
AN antennal nerve, *D* deutocerebrum, *LA* lamina, *LO* lobula, *MBL* medial mushroom bodies lobes, *ME* medulla, *Mu* cibarial muscles, *PL* protocerebral lobes, *RA* retinal axons, *Tr* trachea

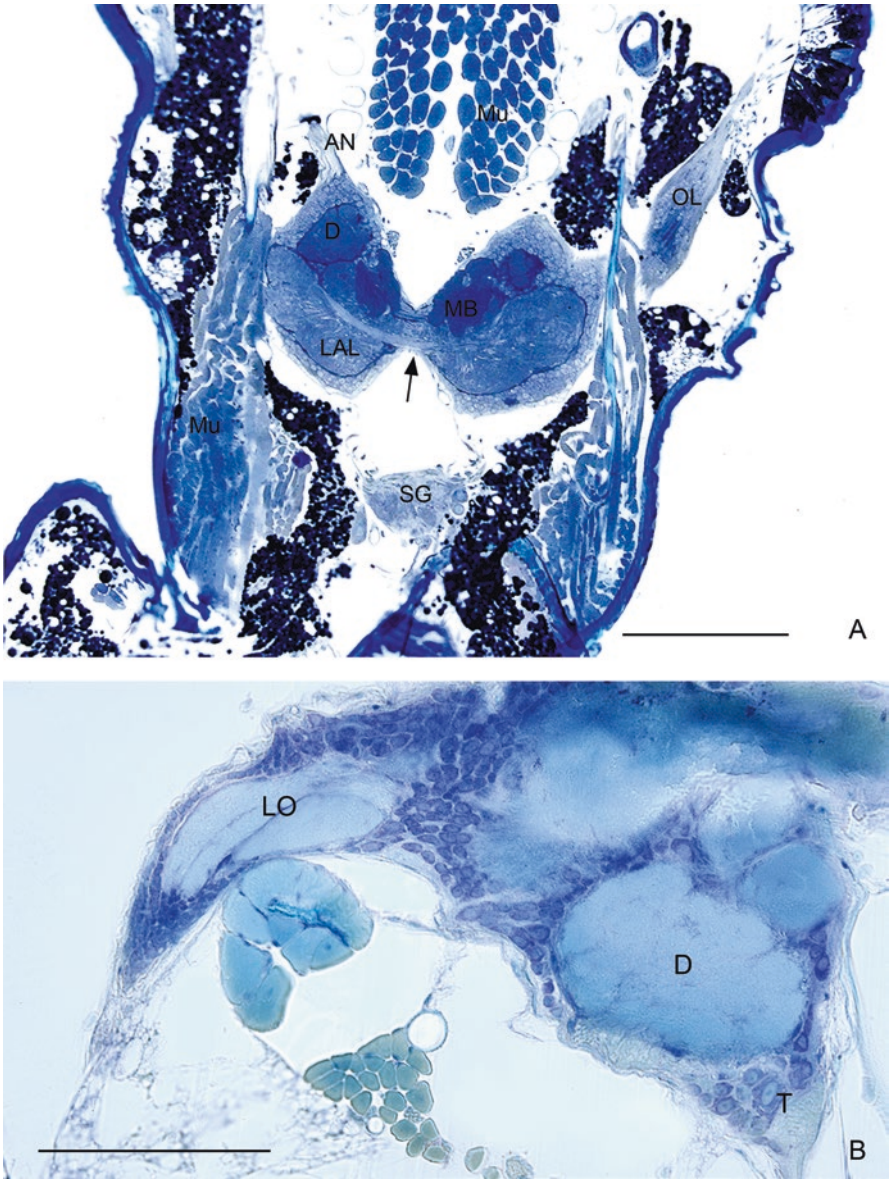

Fig. 11 Light micrographs of sections of the brain of *Rhodnius prolixus*
(**a**) Horizontal section at the level of the lateral accessory lobes of the protocerebrum. The commissure relating both contralateral lobes is shown (arrow). Scale bar 100 μm
(**b**) Frontal section of the brain. Detail of the lobula neuropil of the optic lobe showing the division in three lobes. Scale bar 50 μm
AN antennal nerve, *D* deutocerebrum, *LAL* lateral accessories lobes of the protocerebrum, *LO* lobula, *MB* mushroom bodies, *Mu* muscle, *OL* optic lobe, *SG* subesophageal ganglion, *T* tritocerebrum

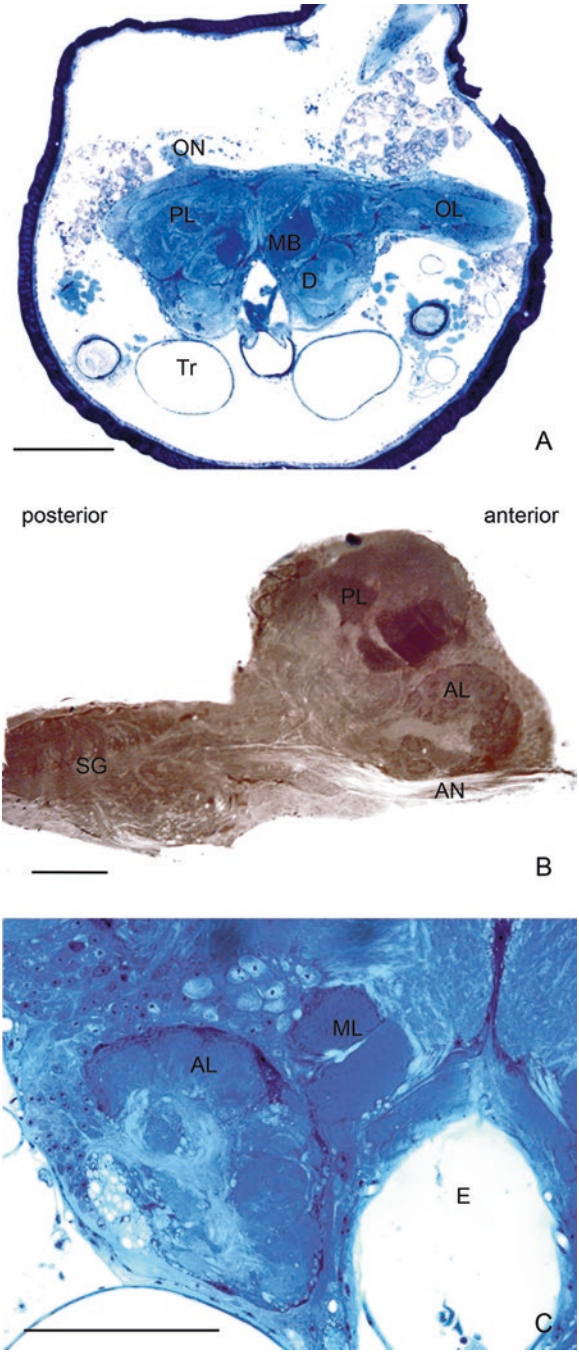

Fig. 12 Light micrographs of sections of the brain of *Rhodnius prolixus* **(a) Frontal section, general view. The main neuropils of the brain are shown. Scale bar 100 µm (b) Horizontal section showing the position of the antennal lobe. Scale bar 100 µm (c) Frontal section with a detail of the deutocerebrum (antennal lobe). Scale bar 50 µm** *AL* **antennal lobe,** *D* **deutocerebrum,** *E* **oesophagus,** *MB/ML* **medial mushroom bodies lobes,** *OL* **optic lobe,** *ON* **ocellar nerve,** *PL* **protocerebral lobes,** *SG* **suboesophageal ganglion,** *Tr* **trachea**

organization, exhibiting 22 glomeruli with a radius of 8–25 μm. No obvious sexual dimorphism of the glomerular architecture was observed.

Tritocerebrum

This is the third and smallest part of the brain in triatomines. It is composed of two small, more or less oval, neuropil masses situated ventral to the antennal lobes on either side of the suboesophageal foramen. Both tritocerebral lobes are connected to each other by a commissure passing beneath the oesophagus (Fig. 13a). A pair of thick fronto-labral nerves arise from the anterior region, connecting with the frontal ganglion. Fibres originating not only in the tritocerebrum but also in the protocerebrum and deutocerebrum, form the circumoesophageal connective, which connect the brain to the suboesophageal ganglion (or gnathal ganglia).

2.3 The Ventral Nerve Cord

The structure of the suboesophageal ganglion and the thoracic ganglia follows the general insect plan. The core is occupied by neuropilar masses, with the cell bodies clustered around.

The suboesophageal ganglion innervates mainly the mandibles, the hypopharynx, the maxillae and the labium. The neuropils corresponding to each segment are amply fused, the most prominent features being the longitudinal tracts and commissures (Fig. 13b).

The prothoracic ganglion is a simple mass with a neuropilar core surrounded by cell bodies (Fig. 13c). The posterior ganglion, is formed by the complete fusion of mesothoracic, metathoracic and abdominal ganglia, but the different neuropilar masses, corresponding to each primitive segment, can be recognized under histological inspection (Fig. 13c).

3 Conclusions and Perspectives

This review summarizes our knowledge on the organization of the nervous system of triatomines. We are far from a representation as detailed as have been obtained in *Musca*, *Drosophila* or *Apis*, for which comprehensive neuroanatomical atlases exist. We think that this kind of work deserves to be deepened for triatomines in order to shed light on the neuroanatomical bases of processes that are increasingly analysed at present, for example, sensory systems, neuromodulation and cognition. Classical histological work and light microscopy appear today as old fashioned methods, but they are still capable of providing extremely fine details about the neural

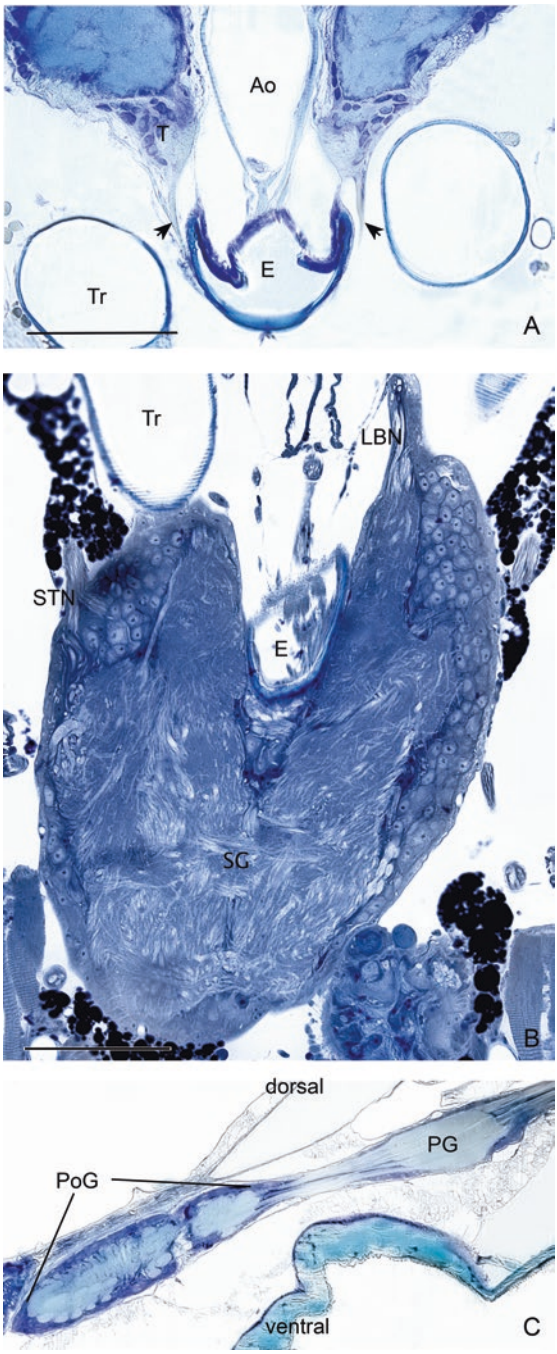

Fig. 13 Light micrographs of sections of the nervous system of *Rhodnius prolixus*
(a) Frontal section showing a detail of the tritocerebrum. Both tritocerebral lobes are connected to each other by a commissure passing beneath the oesophagus (arrows). Scale bar 50 µm
(b) Horizontal section showing the suboesophageal ganglion (gnathal ganglia). The neuropils corresponding to each gnathal segment are amply fused. Scale bar 50 µm
(c) Sagittal section through the thorax showing the ganglionic thoracic mass. In the posterior ganglion, formed by the fusion of mesothoracic, metathoracic and abdominal ganglia, the different neuropilar masses, corresponding to each primitive segment are recognizable. Scale bar 100 µm
Ao **aorta,** *E* **oesophagus,** *LBN* **labial nerve,** *PG* **prothoracic ganglion,** *PoG* **posterior ganglion,** *SG* **suboesophageal ganglion,** *STN* **stylet's nerve,** *T* **tritocerebrum,** *Tr* **trachea**

organization of a study model. Other techniques, including novel neuroanatomical methods may generate valuable information and motivate people to join the effort. An increasing flow of molecular and genetic data is being generated by different laboratories, raising novel functional hypotheses. Some of these ideas need to be placed into a functional context and hypotheses adequately validated. For instance, the relationship between the expression of particular neurotransmitters or neuromodulators in particular regions of the nervous system can only be interpreted in an approximative manner. A detailed atlas would allow establishing links between expression and possible function on a more precise basis.

Another important research need, given the diverse morphological motifs found in this subfamily, is the evaluation of neuroanatomical variation across species. It is worth mentioning that the information presented here is based on the detailed study of two triatomine species, that is, *T. infestans* and *R. prolixus*. It could be observed that the organization of their nervous system is similar, except for small differences in the form (e.g. more or less elongated) and exact position of the brain, constrained by the form of the head capsule of each species. Triatomines being a relatively diversified group in terms of species, habits and head morphology, other differences can be expected to be revealed by future studies.

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Biogenic Monoamines in the Control of Triatomine Physiology with Emphasis on *Rhodnius prolixus*

Angela B. Lange and Ian Orchard

Abstract The biogenic monoamines, serotonin, octopamine, tyramine, dopamine and histamine are present within insect nervous systems, where they modulate multiple and diverse neuronal pathways. In this context, these biogenic amines may be involved in a cascade of events that leads to the initiation and maintenance of a new behavioural act; they bias the nervous and peripheral systems towards a new behavioural state. This review examines the role of these biogenic amines in triatomine physiology, with an emphasis on the most studied triatomine, *Rhodnius prolixus*. Each biogenic amine is reviewed with regard to its biosynthesis and removal, distribution, receptors and physiological relevance, with reference to insects in general and *R. prolixus* in particular. The biogenic amines control various behaviours within *R. prolixus*, including, feeding, reproduction, cuticle tanning and photoreception.

Keywords Nervous system · Biosynthesis · Distribution · Receptors · Physiology

1 Introduction

Biogenic monoamines are a class of organic neuroactive chemicals found in the invertebrates and vertebrates that may act physiologically as neurotransmitters, neurohormones, and/or neuromodulators (for definitions see Orchard 1982, 2009). The biogenic monoamines serotonin, octopamine, tyramine, dopamine and histamine control many physiological and endocrinological processes that are vital to the functioning of an insect.

As discussed previously (Orchard et al. 1993), a cascade of events leads to the initiation and maintenance of a new behavioural act. For example, neurohormones may be released to mobilize energy reserves; muscle properties may be augmented to ensure the generation of the appropriate force; neural circuits are activated to

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generate specific motor patterns; and neuronal circuits may be modulated to adapt the insect to a different set of needs. All these events are part of a complex transition of the insect into a new behavioural state, and a critical aspect of this transition is the need to coordinate these events at the appropriate time; there must be interdependence. This coordination not only depends on neuronal interaction but may also be attributed to neuroactive chemicals that bias, at multiple levels, neuronal, neurohormonal, metabolic and muscular events towards the new functional state of the insect. It is also now apparent that the same neuroactive chemical can be expressed in a variety of cell types, including central and peripheral neurons, neurosecretory neurons, sensory neurons and endocrine cells of the gut; and it is possible that a single neuroactive chemical might represent a functional unit that modulates a common behaviour (e.g. the neuroactive chemical may be the feature shared by the entire signalling pathway involved in a behaviour). Biogenic monoamines are considered to be one such group of chemicals. For example, as we will see later in this chapter, serotonin has a multifunctional role in controlling quite disparate aspects of feeding behaviour in *R. prolixus*, biasing processes towards a new functional state that is initiated by blood gorging.

Norepinephrine and epinephrine are found in low concentrations in invertebrates, and it is unclear if they play a true physiological role in insects and will not be covered here. It would appear that the octopaminergic/tyraminergic signalling pathways in insects have functionally replaced the norepinephrine/epinephrine system of vertebrates. The biogenic monoamines present in insects are distributed throughout the central nervous system (CNS), but may also be found in neurohaemal organs for release into the haemolymph, and in axonal projections going directly to peripheral tissues. They typically act via G-protein-coupled receptors (GPCRs), whose location also identifies target sites for the amine in question.

Here, we review the physiological relevance of biogenic amines in triatomines, with an emphasis on the blood-gorging kissing bug, *R. prolixus.*

2 Serotonin (5-Hydroxytryptamine)

Serotonin, or 5-hydroxytryptamine, is present in both vertebrates and invertebrates and this indicates it to be an evolutionary ancient molecule. Within mammals, serotonin modulates multiple neuronal integrative functions, including mood, anxiety, stress, aggression and feeding. Within insects, including triatomines, it also has a broad range of modulatory activities, including integration of feeding.

2.1 Biosynthetic Pathway and Removal

It is apparent that identical biochemical pathways exist in invertebrates and vertebrates for the synthesis of serotonin from the initial conversion of tryptophan to 5-hydroxytryptophan and then to the final product (Fig. 1). Serotonin is then packaged into granules for subsequent release at synaptic or neurohaemal sites. Within *R. prolixus*, serotonin-like immunoreactive material is found in neurosecretory terminals containing many tightly packed, spherical, electron-dense granules, of approximately 141 nm diameter, ready for release into the haemolymph (Miksys and Orchard 1994).

Vertebrates have two main mechanisms for removing and inactivating biogenic amines from the synaptic cleft; the first is a high-affinity uptake mechanism in which a transporter translocates the amine into the neuron, while the second is via

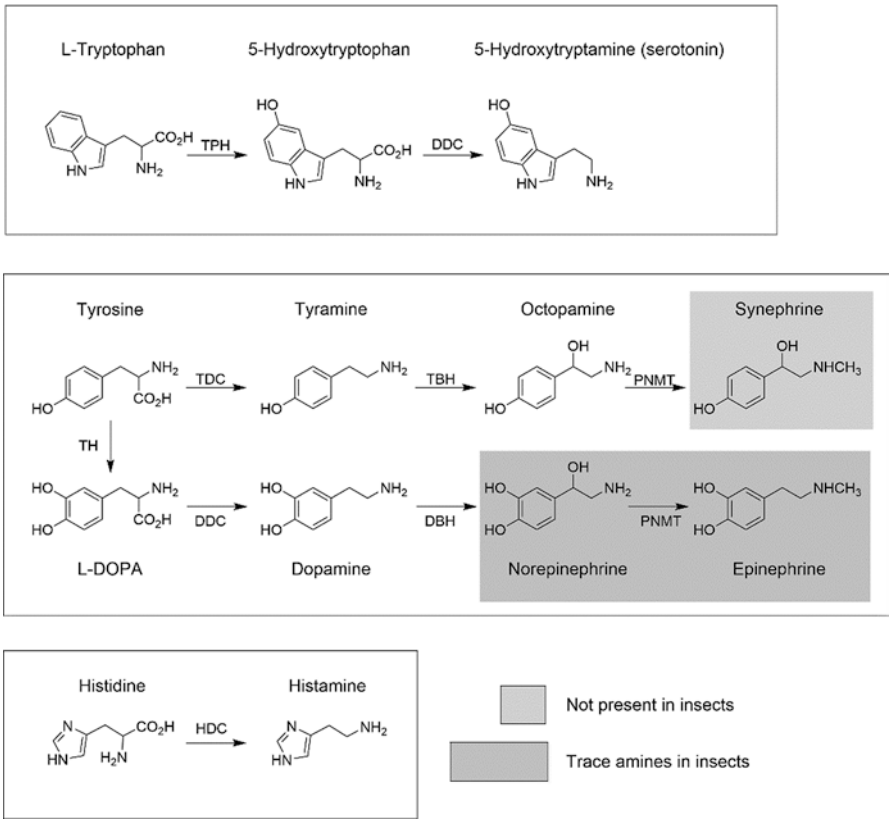


Fig. 1 Biosynthetic pathways of biogenic monoamines. Enzymes: *TPH* tryptophan hydroxylase, *DDC* DOPA decarboxylase, also known as, aromatic amino acid decarboxylase, *TDC* tyrosine decarboxylase, *TBH* tyramine β-hydroxylase, *PNMT* phenylethanolamine N-methyltransferase, *DBH* dopamine β-hydroxylase, *HDC* histidine decarboxylase

degrading enzymes, such as via monoamine oxidase (MAO). Insects do not appear to have large quantities of MAO and it is presumed that an uptake transporter is the primary method of removal of biogenic amines from the synaptic cleft (see Caveny and Donly 2002). The first transporter to be cloned in insects was indeed the *Drosophila melanogaster* serotonin transporter (see Caveny and Donly 2002). The presence of a high-affinity uptake mechanism for serotonin is well documented for the abdominal nerves of *R. prolixus*. These nerves possess serotonergic neurohaemal sites on their surface and the uptake of [^{3}H] serotonin and [^{3}H] tryptophan into these abdominal nerves has been characterized (Orchard 1989). There is a sodium-sensitive, high-affinity uptake mechanism for serotonin and the loaded [^{3}H] serotonin is released in a calcium-dependent manner by high-potassium saline. [^{3}H] tryptophan is also loaded, and 1.4% of this is converted into serotonin over a 3 h incubation. The synthesized [^{3}H] serotonin is also released by high potassium in a calcium-dependent manner from these neurohaemal sites.

2.2 Distribution

Serotonin is widely distributed throughout the insect CNS and peripheral nervous system and its cellular distribution has been mapped in several insect species using specific anti-serotonin antisera. Serotonin is present in interneurons, central neurons with efferent axons, neurosecretory cells and their neurohaemal systems, sensory neurons and the stomatogastric nervous system and gut (see reviews by Nässel 1996; Orchard 2006).

Many insect species possess a bilateral cluster of serotonin-like immunoreactive neurons in the sub-oesophageal ganglion (SOG) which have efferent axons (see Orchard 2006). Some of these project to the salivary glands, whereas others result in neurohaemal sites that are extensive and circuitous, and associated with the nerves of the mouthparts (Bräunig 1987). In *R. prolixus* (see Orchard 2006), serotonin-like efferent neurons in the SOG pass anteriorly towards the mouthparts but then join the salivary nerve and result in fine processes covering the paired salivary glands. Serotonin is also delivered to the salivary glands via the frontal ganglion and recurrent nerve projecting from the oesophagus. Serotonin-like immunoreactivity is found in the corpora cardiaca and extending over the dorsal vessel and digestive tract. Five serotonin-like immunoreactive dorsal unpaired median (DUM) neurons in the abdominal neuromeres of the mesothoracic ganglionic mass (MTGM) project axons through their respective abdominal nerves, resulting in neurohaemal terminals on the abdominal nerves (Orchard et al. 1989). These axons also project over the entire surface of the epidermis on the dorsal cuticle. Serotonin-like immunoreactive DUM neurons are interesting, since as we will see later, most DUM neurons in insects are considered to contain octopamine.

Serotonin-like immunoreactive neurons have also been mapped to the optic lobes and superior protocerebrum in *R. prolixus* (Vafopoulou et al. 2018). Projections travel to the medulla, accessory medulla and lamina, suggesting that serotonin may

be involved in modulation of visual input and also in relaying outputs of the circadian system to the central brain. Serotonin-like immunoreactive neurons have been described in the CNS of *Triatoma infestans* (Settembrini and Villar 2004) where its widespread distribution suggests it to function as a neuromodulator or as a neurohormone.

2.3 Receptors

Serotonin GPCRs, divided into 3 classes of receptors, have been identified and characterized in a number of insects (see Roser et al. 2012; Ngai et al. 2019). In *R. prolixus*, a serotonin type 2b receptor, Rhopr5HTR2b, has been cloned and characterized (Paluzzi et al. 2015). The total length of the Rhopr5HTR2b cDNA amplified is 2299 bp, with no additional splice variants identified. The gene spans over 80.7 kb and includes six exons and five introns. The single open reading frame (ORF) of 2109 bp produces a protein of 703 amino acid residues. In common with other members of the GPCR superfamily, the deduced protein sequence contains seven hydrophobic transmembrane domains. The amino-terminus (N-terminus) has a length of 16 amino acids while the carboxyl terminus (C-terminus) length is 25 amino acids. The ORF begins on the second exon, which also yields the first and second transmembrane domains. The third and fourth transmembrane domains are localized to the third exon while the fifth hydrophobic domain is found on the fourth exon. The fifth exon lacks any predicted transmembrane domains but yields the N-terminal region of the third intracellular loop while transmembrane domains six and seven are localized on the sixth and final exon.

The Rhopr5HTR2b transcript is enriched in the CNS, Malpighian tubules, salivary glands and dorsal vessel. A heterologous functional receptor assay illustrates that Rhopr5HTR2b is dose-dependently activated by serotonin with an EC_{50} in the nanomolar range. Rhopr5HTR2b is sensitive to alpha-methyl serotonin and inhibited by a variety of serotonin receptor antagonists, including propranolol, spiperone, ketanserin, mianserin and cyproheptadine. In contrast, the cardioacceleratory activity of serotonin reveals a different pharmacological profile, with no significant response induced by alpha-methyl serotonin and insensitivity to ketanserin and mianserin. This distinct agonist/antagonist profile indicates that a separate serotonin receptor type may mediate cardiomodulatory effects controlled by serotonin in *R. prolixus*. This, along with the fact that Rhopr5HTR2b is not enriched in the anterior midgut where serotonin stimulates absorption and elicits myotropic control, indicates that there is likely more than one serotonin receptor in *R. prolixus*. A recent survey of *R. prolixus* confirms this, where 5 serotonin GPCRs have been annotated (Ons et al. 2016).

Serotonin receptors in *R. prolixus* appear to couple through the adenylate cyclase second messenger pathway and increases in cyclic AMP (cAMP) content of many peripheral target tissues have been shown in response to serotonin (see Orchard 2006; Orchard and Lange 2019). In the salivary glands of *Calliphora vicina*, two

sub-types of serotonin receptors are present, one of which couples to the phospholipase C pathway and the other to the adenylate cyclase pathway. A complex functional crosstalk between these pathways leads to the transport of ions and water across the salivary gland epithelia (Roser et al. 2012).

2.4 Physiological Relevance of Serotonin in R. prolixus

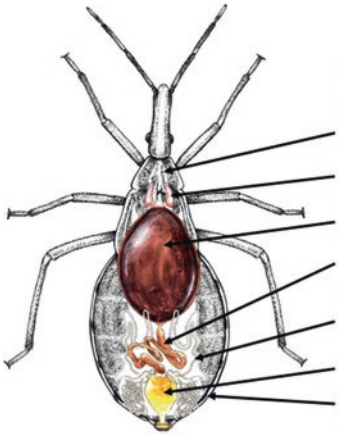
Serotonin as a Neurohormone

During gorging, fifth instar *R. prolixus* release serotonin from abdominal nerve neurohaemal areas, resulting in a dramatic rise in the titre of serotonin in the haemolymph from an unfed level of about 7 nM to a peak of 115 nM reached 5 min after the onset of gorging (Lange et al. 1989). The concentration of serotonin then declines considerably by the time gorging is completed. These findings lead to the inevitable conclusion that serotonin is a neurohormone in *R. prolixus* released by the natural stimulus of feeding (Lange et al. 1989).

Coordination of Feeding

The coordination of insect feeding in general is not well understood, particularly because of the complexity of events associated with both external and internal signals. *R. prolixus* has afforded some advantages in this endeavour; much is known about the feeding behaviour, gorging stimulus and feeding apparatus in this insect (see Smith 1985; Latorre-Estivalis and Lorenzo 2019), and considerable information is available regarding the control of salt and water balance (see Coast et al. 2002; Orchard 2006; Orchard and Lange 2019).

Serotonin regulates feeding activities in a variety of invertebrates (see Orchard 2006) and many of the known functions for serotonin in *R. prolixus* fit into a scheme whereby serotonin coordinates feeding-related physiological events (Fig. 2), as it appears to do so in another blood-feeding vector, *Aedes aegypti* (see Ngai et al. 2019). Serotonin 'orchestrates' these activities either by direct delivery to target tissues within the nerve supply (as a neurotransmitter / neuromodulator) and/or by transport in the haemolymph as a neurohormone (Fig. 2). During feeding in *R. prolixus*, the content of serotonin decreases within the processes projecting over target tissues and within neurohaemal sites, and concurrently increases in the haemolymph. Serotonin, as a neurotransmitter/neuromodulator and/or as a neurohormone, acts upon many tissues involved with blood-gorging. Serotonin stimulates salivary gland muscle contraction and secretion, stimulates contraction of digestive tract muscles (anterior midgut and hindgut), enhances heart-beat, induces plasticization (stretching) of the cuticle to accommodate the massive blood meal, increases the expression of aquaporins in Malpighian tubules, stimulates water and ion movement from the anterior midgut into the haemolymph, stimulates transcript



Tissue	IR processes	R	Increases cAMP	Biological effect
Dorsal Vessel	✓	✓	ND	Stimulates an increase in heartbeat frequency
Salivary Glands	✓	✓	✓	Stimulates secretion and muscle contraction
Anterior Midgut	✓	X	✓	Stimulates fluid transport and muscle contraction
Posterior Midgut	✓	X	ND	Stimulates expression of acyl-CoA-binding protein
Malpighian Tubules	X	✓	✓	Stimulates secretion (upper MT) and absorption (lower MT) and stimulates expression of aquaporin
Hindgut	✓	✓	✓	Stimulates muscle contraction
Epidermis of cuticle	✓	X	✓	Stimulates plasticization

Fig. 2 The involvement of serotonin with various tissues involved in feeding in *R. prolixus*. Serotonin-like immunoreactive (IR) processes innervate most tissues, the Rhopr5HTR2b serotonin receptor (R) is present in most tissues, and serotonin typically increases cAMP resulting in a biological effect. *ND* not determined. In the cartoon, the digestive system has been mapped into the outline of a fifth instar *R. prolixus*. The anterior midgut has been displaced anteriorly to expose the posterior midgut and Malpighian tubules (MT). (See Orchard and Lange 2019 for references)

expression of acyl-CoA-binding protein (ACBP) in posterior midgut (influences lipid metabolism), and stimulates water and ion movement across the Malpighian tubules (see Orchard 2006, 2009; Orchard and Lange 2019; Alvez-Bezerra et al. 2010; Majerowicz and Gondim 2013). This latter, very rapid, diuresis is particularly essential since each instar of *R. prolixus* consumes one blood meal that can be up to ten times its initial body mass. This severely restricts the mobility of the insect and it is essential that much of the fluid load (particularly water and salt) be voided as rapidly as possible. Thus, *R. prolixus* begins to urinate even before it has finished the blood meal, a process controlled by the rapid actions of serotonin.

It is possible that other activities might be influenced by serotonin, although these have yet to be tested (e.g. host-seeking behaviour, initiation of gorging, satiation); although it has been suggested that the satiety level, interrelated with the serotonin titres, might influence the response to heat (Zhukovskaya and Polyanovsky 2017; Bodin et al. 2009).

Although this single neuroactive chemical seems to orchestrate feeding behaviour in *R. prolixus* it does not necessarily act alone on any given target tissue. Thus, a number of target tissues that are responsive to serotonin also receive input from members of a variety of neuropeptide families, including, for example, the kinins, corticotropin-releasing factor/diuretic hormones, tachykinins, FMRF-like peptides and CAPA (see Orchard and Lange 2019). Some of these effects may be additive; others may be synergistic; the anti-diuretic hormone CAPA can be inhibitory on the effects of serotonin. Some involve a complex interplay between second messengers (see Gioino et al. 2014; Lee et al. 2016; Orchard and Lange 2019). Also, the time courses over which the variety of neurochemicals act may be different, with

serotonin eliciting rapid onset of effects, and neuropeptides eliciting longer term effects.

Salivary Secretions

Triatomes have evolved complex salivary secretions that can help overcome the physiological defence mechanisms of the host (Andersen et al. 2005). One class of salivary gland proteins in *R. prolixus* are amine-binding proteins. These were first suspected since *R. prolixus* saliva prevents serotonin-stimulated contractions of the rat uterus. Isolation of this lipocalin and subsequent analysis reveals that the lipocalin acts by binding to serotonin (and also norepinephrine), preventing them from binding to their receptors in the host; and hence the name, amine-binding protein.

3 Octopamine (OA)

The monoamine D-octopamine (OA) is a neuroactive chemical that was first discovered in the salivary gland of the octopus, *Octopus vulgaris* (Erspamer 1948), and hence its name. It is one of the most abundant biogenic amines in the nervous system of invertebrates and has been the subject of intense investigations (see Orchard 1982; Sotnikova and Gainetdinov 2009; Farooqui 2012). In invertebrates, OA functions as an important neurotransmitter, neuromodulator and neurohormone. It is believed that OA plays a pleiotropic role in the physiology of invertebrates similar to that seen for epinephrine in vertebrates. Octopamine is structurally and functionally similar to norepinephrine of vertebrates (Orchard 1982; Roeder 1999; Bauknecht and Jékely 2017).

3.1 Biosynthetic Pathway and Removal

Octopamine is synthesized from the amino acid tyrosine via a two-step process. Decarboxylation of tyrosine by tyrosine decarboxylase (TDC) results in the production of tyramine (TA) (Fig. 1). Hydroxylation of tyramine by tyramine β-hydroxylase converts TA to OA by the addition of a hydroxyl group. Re-uptake is the most important mechanism of inactivation for this type of small transmitter compound and is achieved by specialized transporter molecules (see Caveny and Donly 2002). In contrast to vertebrates, MAO plays only a minor role in the inactivation of monoamines in invertebrates and it is thought that inactivation of OA occurs predominantly via other mechanisms such as N-acetylation and N-methylation. All the functional elements necessary for packaging of monamines into granules for subsequent storage and release, release-related events, and reuptake of released monoamines via plasma membrane transporters have been identified for octopaminergic

transmission in a variety of insects (see Caveney and Donly 2002), although no work has been done as yet in *R. prolixus*.

3.2 Distribution

Octopamine-containing neurons are only a small subset of the neurons (40–100 cells) within the nervous system of insects. Typically, although not exclusively, OA is synthesized in unpaired median neurons of insects whose cell bodies are located either ventrally (ventral unpaired median neurons or VUM neurons) or DUM neurons in the SOG and ventral nerve cord (VNC) (Bräunig and Borrows 2004; Sinakevitch et al. 2005). It has also been reported that unpaired median neurons of the thoracic ganglion send efferents to most organs and muscles, whereas those of the SOG broadly innervate nearly all parts of the brain (Bräunig and Borrows 2004). In the locust, *Schistocerca gregaria* and the cockroach, *P. americana*, OA is also found in the optic lobes as well as associated with the corpora cardiaca (Evans 1978). There are no published reports on the distribution of octopaminergic neurons in triatomines although DUM neurons that are OA-like immunoreactive are present in the MTGM (Lange and Orchard, unpublished).

3.3 Receptors

Octopamine mediates its effects by binding to GPCRs that share significant structural features with vertebrate adrenergic receptors and are therefore classified as α-adrenergic-like receptors, β-adrenergic-like receptors and OA/TA receptors, a class of receptors that binds both OA and TA with OA binding preferential to the receptor. The first receptor cloned and characterized was a *D. melanogaster* TA receptor shown to negatively couple to adenylate cyclase (Arakawa et al. 1990). Fast forward 29 years later, many OA and TA receptors have been cloned and characterized from different insect orders, most notably in Diptera, Lepidoptera and Hymenoptera (Ohta and Ozoe 2014; Wu et al. 2015; Reim et al. 2017). The activation of a specific receptor leads to a unique change(s) in cAMP and/or Ca^{2+}.

In *R. prolixus,* RhoprOctb2-R (MF377526), an OA receptor with similarities to other insect Octb2 receptors has been cloned and sequenced and the ORF spans four exons separated by three intronic regions (Hana and Lange 2017a). RhoprOctb2-R belongs to the rhodopsin-like (Class A) GPCR superfamily characterized by seven transmembrane hydrophobic domains (TM), a DRY residue in the TM3, and an NPxxY motif in TM7. RhoprOctb2-R was transiently expressed in HEK293/CNG cells and the interaction of the ligand with the receptor was monitored by measuring the bioluminescence released due to calcium mobilization in the cytosol. RhoprOctb2-R was activated in a dose-dependent manner by both OA and TA, with TA (EC_{50} = 3.85 × 10^{-6} M) ten times less potent than OA (EC_{50} = 3.67 × 10^{-7} M).

Octopamine has also been shown to be one order of magnitude more potent than TA in *Nilaparvata lugens* (Wu et al. 2017), *Apis mellifera* (Balfanz et al. 2014) and *D. melanogaster* (Maqueira et al. 2005). Octopamine fully activates the receptor, unlike TA which is a partial agonist. Pharmacological analysis using a variety of receptor antagonists indicates that phentolamine and gramine significantly reduce the ligand-induced luminescence response, suggesting a pharmacological profile similar to other Octb2-receptors in insects (Hana and Lange 2017a). An in silico study of the GPCRs in *R. prolixus* has annotated 4 OA and 1 OA/TA receptor (Ons et al. 2016).

Octopamine acts via cAMP to decrease the amplitude of spontaneous oviduct contractions (see section below; Hana and Lange 2017b). These physiological data complement the receptor data that reveal RhoprOctb2-R likely couples to a Gs protein leading to the activation of adenylate cyclase and elevation of intracellular cAMP in the HEK293/CNG cells used in the functional receptor assay (Hana and Lange 2017a).

3.4 Physiological Relevance of OA in R. prolixus

Octopamine is present in relatively high concentrations in both neuronal and non-neuronal tissues of most invertebrate species studied and has been shown to have a plethora of functions. Octopamine acts as a stress hormone ('flight or fight' hormone), adjusting the body to the animal's energy demands when needed (Orchard et al. 1993). In the peripheral nervous system, OA modulates the activity and energy metabolism of flight muscles, peripheral organs (such as fat body, oviduct and haemocytes), and almost all sense organs (see Orchard et al. 1993; Adamo et al. 1995). It has been shown to play roles in promoting aggression and wakefulness in flies, modulation of locust flight, neural control of reproductive tissues in the locust, and learning and memory (Orchard 1982; Orchard et al. 1993; Farooqui 2012; Ohta and Ozoe 2014; Roeder 1999; Bauknecht and Jékely 2017).

In *R. prolixus*, OA plays an important role in the ability of mature fed and mated females to make and lay eggs. Octopamine decreases the amplitude of spontaneous and peptide-induced contractions of the oviducts and reduces the frequency of bursal contractions, indicating that it may inhibit the passage of eggs along the reproductive tissue during egg-laying (Hana and Lange 2017b). The movement of eggs during egg laying in *R. prolixus* requires oviducal and bursal peristaltic and phasic contractions for successful fertilization and deposition (Sedra and Lange 2014). This ability of OA to inhibit reproductive tissue contractility is consistent with results obtained in *Locusta migratoria*, *D. melanogaster* and *Stomoxys calcitrans* (see Sedra and Lange 2014). In *D. melanogaster,* OA is also necessary to trigger ovulation (Lee et al. 2003; Monastirioti 2003).

Phentolamine, an α-adrenergic receptor antagonist, attenuates the inhibition induced by OA on oviduct and bursal contractions in *R. prolixus* (Hana and Lange 2017a). Phentolamine has been shown to be an effective OctβR antagonist in the

oviducts of *L. migratoria* (Lange and Orchard 1986; Orchard and Lange 1986). Octopamine also increases the levels of cAMP in the oviducts; an increase which is blocked by phentolamine, suggesting that OA may act on the reproductive tissue via an Octβ receptor leading to inhibition of muscle contraction. The physiological implications of these findings in *R. prolixus* are that OA plays an essential role in the process of ovulation. Relaxation of the oviducts allows the ovary to release more eggs into the oviducts. In *D. melanogaster*, OA relaxes the oviducts, thereby enabling the release of eggs into the oviducts (Middleton et al. 2006). Moreover, Octβ2R and OAMB (OA receptor expressed primarily in the mushroom bodies) have been found to be the receptors associated with the process of ovulation and fertilization in *D. melanogaster* (Lee et al. 2003; Li et al. 2015). Deletions in the OAMB locus and mutant constructs of Octβ2R result in accumulation of eggs in the ovary and a significant decrease in the number of eggs laid (Lee et al. 2003; Li et al. 2015).

The transcript of RhoprOct2β-R is enriched in the CNS and is expressed throughout tissues of the adult female reproductive system, including the oviducts and bursa (Hana and Lange 2017a). Knockdown experiments in *D. melanogaster* indicate that Octβ2-R is essential for ovulation and fertilization. Insects that specifically lack OA (tyramine β-hydroxylase mutants) accumulate eggs in their ovaries due to an absence of ovulation (Monastirioti 2003). RhoprOctβ2-R is highly expressed in the oviducts of *R. prolixus* relative to other reproductive tissues (Hana and Lange 2017a). RhoprOctβ2-R is a key component of octopamine signalling in the lateral oviducts and this receptor could be responsible for the physiological effects seen at the bursa. In *D. melanogaster* and *N. lugens*, mutated Octβ2-R causes an accumulation of eggs in the ovary and eliminates egg fertilization in *D. melanogaster* (Li et al. 2015; Wu et al. 2017).

Interestingly, there is evidence for OA receptors in developing ovarian follicles in *R. prolixus* (O'Donnell and Singh 1989). This study was the first to report on the modulation of excitable properties of an egg cell by a neurotransmitter. Ovarian follicles of *R. prolixus* produce action potentials of 2–3 s duration when depolarized and OA decreases electrical excitability of the follicles. These effects are most likely mediated through an increase in the intrafollicular concentration of cAMP.

4 Tyramine (TA)

As mentioned above, tyramine (TA) is the precursor of OA and therefore demonstration of the role of TA as a neurotransmitter, neuromodulator, or neurohormone has been difficult; however, it is believed that both OA and TA function analogously to epinephrine and norepinephrine in vertebrates (Roeder 2005). Interestingly, in terms of structure, TA more resembles DA (Fig. 1). Recent studies have shown that TA can display different effects than OA, and thus TA is now considered to be a neuroactive chemical in its own right, independent of OA (Kononenko et al. 2009; Lange 2009).

4.1 Biosynthetic Pathway and Removal

Tyramine is synthesized via the decarboxylation of the amino acid tyrosine via TDC (Lange 2009) (Fig. 1). In *D. melanogaster*, TDC 2 is responsible for synthesizing TA in neuronal cells, whereas TDC 1 synthesizes non-neuronal TA in peripheral tissues (Cole et al. 2005). Conversion to OA leads to degradation via a number of mechanisms including N-acetylation, N-methylation, potential MAO-mediated inactivation (although minor) and β-alanine conjugations (Farooqui 2012).

4.2 Distribution

In the CNS of the locust, the neuronal content of OA is 3–7 times higher than that of its precursor TA (Lange 2009). The distribution of TA-specific neurons, those that do not also contain OA, has been described in a variety of insects. In *S. gregaria*, immunoreactive TA neurons are found in the medulla, the protocerebral bridge, the antennal lobes, SOG and associated neuropiles (Kononenko et al. 2009). In *D. Melanogaster* larvae, TA-specific neurons are found in the brain neurons, thoracic ganglia and abdominal ganglia (Monastirioti et al. 1996). Tyramine-containing neurons, which also contain OA, have been found in the SOG and thoracico-abdominal ganglia (Nagaya et al. 2002). These neurons are characterized as VUM neurons, except for in the terminal abdominal ganglion where the TA-containing neurons are DUM neurons (Nagaya et al. 2002). VUM and DUM neurons, which also contain OA, innervate skeletal muscles and other peripheral target tissues (Lange 2009). No studies have been published on the distribution of tyraminergic neurons in triatomines.

4.3 Receptors

In *R. prolixus*, a TA receptor, RhoprTyr1-R, has been cloned and sequenced, and shares key structural features in common with other Tyr1-Rs in other insects (Hana and Lange 2017a). The RhoprTyr1-R ORF spans a single exon with a length of 1526 bp. Characteristic to all Tyr1-Rs in insects, the third intracellular loop of RhoprTyr1-R (151 amino acids) is elongated when compared to RhoprOctb2-R's (70 amino acids) third intracellular loop. RhoprTyr1-R preferentially binds TA, with the ligand TA ($EC_{50} = 5.17 \times 10^{-8}$ M) being two orders of magnitude more potent than the ligand OA ($EC_{50} = 6.88 \times 10^{-6}$ M) in activating RhoprTyr1-R. Nonetheless, OA fully activated Tyr1-R at concentrations $\geq 10^{-4}$ M. In a similar manner, Tyr1-Rs from *Bombyx mori* (Ohta et al. 2003) and *L. migratoria* (Vanden Broeck et al. 1995) are two orders of magnitude more sensitive to TA than OA (Vanden Broeck et al. 1995; Ohta et al. 2003). In *A. mellifera* (Blenau and Baumann 2001) and *D.*

melanogaster (Enan 2005), TA is only one order of magnitude more potent than OA. Yohimbine is the most effective antagonist in inhibiting TA's activation of RhoprTyr1-R in the functional receptor assay using HEK-293/CNG cells. Overall, yohimbine has been established as the most potent antagonist of insect Tyr1-Rs (Vanden Broeck et al. 1995; Enan 2005; Sotnikova and Gainetdinov 2009). RhoprTyr1-R likely couples to a Gq protein leading to the release of Ca^{2+} from intracellular stores through the IP_3 pathway (Hana and Lange 2017a).

Analysis of Tyr1-R transcript distribution reveals that TA receptors are highly expressed in the CNS (Blenau et al. 2000; El-Kholy et al. 2015), strongly expressed in the heart, with minor expression in the reproductive organs (El-Kholy et al. 2015). Similar to other insects, strong expression of RhoprTyr1-R transcripts is detected in the CNS relative to the lower transcript expression in the oviducts of *R. prolixus*. Lower RhoprTyr1-R transcript expression in the oviducts, relative to other reproductive tissues, reinforces the fact that TA is unable to inhibit oviduct contractions by itself (Hana and Lange 2017a, b). RhoprTyr1-R transcript expression in the lateral oviducts could signify TA as a neuromodulator rather than a neurotransmitter at this tissue (Hana and Lange 2017a). In this scenario, TA modulates the activity of other neuropeptides that stimulate contraction rather than directly influencing contraction (see section below).

4.4 Physiological Relevance of TA in R. prolixus

Physiologically, TA, along with its metabolic precursor, OA, has often been shown to have similar effects in invertebrates (Roeder 1999, 2005). An accumulating amount of evidence indicates that TA can exhibit its own physiological actions independent of OA (Lange 2009). A role for TA has also been confirmed by the discovery of TA-containing neurons (without OA) and the presence of TA-specific receptors. For example, elevation of TA attenuates locomotion in *D. melanogaster* larvae lacking OA and feeding OA rescues locomotion in mutant flies (Saraswati et al. 2004). Tyramine has also been shown to be involved in the regulation of many physiological processes, such as reproduction, aggression, feeding and locomotion (see Ohta and Ozoe 2014; Farooqui 2012). A tyramine 1 (Tyr1) receptor in *L. migratoria*, the octopamine receptor in the mushroom bodies (OAMB), has been linked to reproductive physiology (Lee et al. 2003; Donini and Lange 2004; Molaei et al. 2005; Li et al. 2015).

In *R. prolixus* the role of both OA and TA in modulating myogenic contractions of the oviducts and the bursa of the adult female has been investigated (Hana and Lange 2017b). Interestingly, TA and OA have differing effects on the oviducts, with OA inhibiting muscle contraction in a dose-dependent manner and TA having no effect on the amplitude of contraction; however, both amines are capable at inhibiting a peptide-induced contraction. This is not the case in *L. migratoria*, where TA mimics OA and decreases the amplitude of contraction, the basal tonus and attenuates proctolin-induced contractions (Donini and Lange 2004). In *R. prolixus,* these

results suggest that OA acts as a neurotransmitter at the oviducts leading to inhibition of contraction via a cAMP signalling pathway, whereas TA regulates oviduct muscle contraction via a modulatory mechanism altering contractile properties of a stimulated muscle. Interestingly, the effects of OA and TA on the bursa are similar. Both biogenic amines completely abolish contractions of the bursa most likely via inhibiting a pacemaker (Hana and Lange 2017b). Phentolamine, known to block α-adrenergic receptors, antagonizes the effects of OA and TA at the bursa, suggesting that both amines likely act via the same receptor. In support of these data, yohimbine, a TA receptor blocker, does not antagonize the effects of TA and OA on the bursa. This suggests that TA acts via the RhoprOctβR at high concentrations, as shown in the locust oviducts and foregut (Britain 1990; Donini and Lange 2004).

5 Dopamine (DA)

Dopamine (DA) is present throughout all organisms (including vertebrates and invertebrates) and so is an evolutionary ancient molecule. Dopamine modulates many neuronal pathways in animals, including appetitive learning, social interactions, arousal and sclerotization and melanization in insects (Verlinden 2018).

5.1 Biosynthetic Pathway and Removal

Dopamine synthesis in invertebrates is similar to vertebrates (see Verlinden 2018) with the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme, tyrosine hydroxylase (TH) and subsequent conversion of L-DOPA to L-DA by DOPA decarboxylase (Fig. 1). In *D. melanogaster* DA is inactivated by the enzyme β-alanyl-DA-synthetase or by N-acetyltransferase which converts DA to N-acetyl-DA (Verlinden 2018). There is some evidence that there are DA transporters in insects, and these transporters have been cloned in a few insect species (see Caveny and Donly 2002), but not in triatomines.

5.2 Receptors

G-protein coupled receptors for DA have been identified and characterized in a variety of insects, and classified into 4 types which may couple to the PLC or adenylate cyclase pathway or both (see Verlinden 2018). No DA receptors have been cloned from triatomines, but in silico analysis has annotated 4 DA receptors in *R. prolixus* (Ons et al. 2016).

5.3 Distribution

The distribution of DA neurons within the CNS has been mapped using immunohistochemistry, utilizing antisera against DA or against the rate-limiting enzyme in its biosynthetic pathway, TH (see Tedjakumala et al. 2017; Mesce et al. 2001). Dopaminergic interneurons are found throughout many regions of the brain and VNC of insects, and efferent dopaminergic neurons control activities of the salivary glands (Ali 1997). Within *R. prolixus* each thoracic ganglia of the VNC contains a single, large VUM neuron which stains for TH-like immunoreactivity and for catecholamines (Orchard 1990). These have distinct axonal branching patterns that remain within the CNS. They resemble the H cell of Goodman et al. (1981); later shown to contain DA (Mesce et al. 2001). A small number of paired neurons are also observed within each ganglion of the VNC of *R. prolixus*, again with axons that remain within the CNS. A large number of TH-like immunoreactive neurons are found in the optic lobe, with about 160 neurons elsewhere in the brain, distributed over both ventral and dorsal brain and largely in the protocerebrum of the CNS (Nyhof-Young and Orchard 1990). The wide distribution suggests DA neurons with diverse central functions.

5.4 Physiological Relevance of DA in R. prolixus

Dopamine has been shown to play modulatory roles in many behaviours in insects, including feeding behaviour (and control of salivary glands), aversive learning, arousal, social interactions, reproductive physiology, locust phase change, and cuticle scleratization and melanization (see Verlinden 2018). Within *R. prolixus* DA has not been extensively studied but has been examined in a small number of studies.

Reproductive Physiology

Dopamine is a neurotransmitter that mediates the effects of 20-hydroxyecdysone (20-HE) on neurosecretory cells in the brain of *R. prolixus* (Orchard et al. 1983). The timing and rate of oviposition in *R. prolixus* is controlled by a myotropic ovulation hormone released from neurosecretory cells in the brain. In mated females, the electrical activity of these neurons and associated release of hormone are stimulated by the appearance of 20-HE in the haemolymph. The electrical activity recorded during times of hormone release is very characteristic, and involves a phasic pattern of short bursts of action potentials (Ruegg et al. 1982). This activity can be induced in an isolated head preparation from an ovariectomized female by application of 10^{-7} M 20-HE (Ruegg et al. 1982). Dopamine, at 10^{-7} M, mimics the effects of 20-HE. The effects of 20-HE and DA are each antagonized by the aminergic receptor antagonist, phentolamine. Thus, the effects of 20-HE on the neurosecretory cells

are indirect, and involves aminergic interneurons, possibly those containing DA (Orchard et al. 1983).

Feeding-Related Activities

In contrast to the effects of serotonin shown earlier, DA does not stimulate secretion from Malpighian tubules or absorption across the anterior midgut (Farmer et al. 1981; Maddrell et al. 1971). Whilst DA does stimulate plasticization of cuticle, it is 500 times less potent than serotonin, and so this is likely due to cross-reactivity with a serotonin receptor (Reynolds 1974).

Cuticle

Melanin is synthesized from DA in insects, and cuticle tanning, which occurs after hatching or ecdysis is due to melanin deposition and protein cross-linking. Disruption of this pathway in *R. prolixus* (by silencing the enzyme DOPA decarboxylase) results in reduced nymphal survival with nymphs dying around the time of ecdysis (Sterkel et al. 2019). Those nymphs that survive ecdysis have cuticles with reduced pigmentation and reduced hardening. The eggs from adult females with silenced enzyme have reduced hatching and those that do hatch produce nymphs that fail to tan or feed (Sterkel et al. 2019).

6 Histamine (HA)

Histamine ('tissue' amine, HA) was discovered in 1910 by Sir Henry Dale. It is present in vertebrates and invertebrates. In the vertebrates, HA is a neuroactive chemical in the nervous system, and a signalling molecule in the gut, skin and immune system. In insects, HA is predominantly found in retinal photoreceptors and optic lobe where it projects to clock neurons.

6.1 Biosynthetic Pathway and Removal

A single enzymatic step converts L-histidine to HA in both invertebrates and vertebrates (Fig. 1). Histamine is inactivated by metabolism to carcinine by the same enzyme that converts DA to β-alanyl-DA (Denno et al. 2016).

6.2 Distribution

Histamine content in insects is highest in the eyes, with much lower levels elsewhere in the CNS (Denno et al. 2016). Histamine is the neurotransmitter of photoreceptors in insects and other arthropods, and acts on ligand-gated chloride channels (Nässel 1999; Akashi et al. 2018). In addition to its presence in photoreceptor cells, HA is also found in a relatively small number of neurons in the insect CNS, and hence the low overall content. These histaminergic neurons project to many areas of the brain suggesting broad activity in a variety of physiological responses. It is suggested that the histaminergic neurons may form wide field inhibitory systems which might be distinct from those neurons using the inhibitory GABA (Nässel 1999). No studies have reported the distribution of HA in triatomines.

6.3 Receptors

No GPCRs for HA have been identified in insects, and none have been identified in an in silico analysis of the *R. prolixus* genome (Ons et al. 2016).

6.4 Physiological Relevance of HA in R. prolixus

Salivary Secretions

Other than HA's involvement in vision, there are a few studies on HA in insects and none in triatomines. Interestingly, though, the saliva of triatomines, as well as that of other blood-feeding insects, contains a large variety of pharmacologically active components, including those with anti-histaminergic activity. Nitrophorins tightly bind HA with a high affinity and are therefore responsible for this anti-histaminergic activity in *R. prolixus* and other triatomines (Andersen and Ribeiro 2017). Nitrophorin is referred to as a kratagonist.

7 Concluding Remarks

Biogenic amines play a vital role in the physiology/endocrinology of triatomines, as demonstrated by their activities in *R. prolixus.* Within *R. prolixus*, each amine is distributed in its own unique neuronal pattern, and may be present in interneurons, efferent neurons, neurosecretory neurons, sensory neurons, or variations thereof. They can certainly act as neurotransmitters, neuromodulators and/or neurohormones. We know a considerable amount about GPCRs for serotonin, OA and TA,

but more need to be characterized. We know little about GPCRs for DA, or the ion channels for HA.

Whilst serotonin controls many feeding-related activities, especially those involving the periphery, little is known about serotonin's central effects. In addition, there is much to be understood about serotonin's interactions with neuropeptides.

Similarly, OA and TA have only just begun to be investigated in any detail, and again, little is known about their central effects, and much more needs to be discovered about their involvement in reproduction and other behaviours.

Dopamine has been even less studied in *R. prolixus*. It is distributed in a relatively small number of neurons within the CNS, but other than its involvement with cuticle tanning, little or no studies have been performed on its physiological relevance. In light of DA's importance in other insects, future studies on DA in *R. prolixus* are certainly warranted.

Histamine has not been studied in *R. prolixus* (or any other triatomines). Since it appears to be the transmitter involved in photoreception, controlling brain clock neurons, it is particularly important to study HA in *R. prolixus* which has circadian rhythms associated with feeding, locomotion, hormone release, ecdysis and egg laying.

In conclusion, whilst much is known about biogenic amines in triatomines, there is much more to be discovered.

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Structure and Physiology of the Neuropeptidergic System of Triatomines

Sheila Ons and Marcos Sterkel

Abstract In insects, neuropeptides can act as hormones and neurotransmitters, exerting their physiological roles by activating membrane protein receptors. Because of their relevance in the control of vital processes, the neuroendocrine system of harmful insects could be a potential source of targets for next-generation insecticides, in an integrated and environmentally sustainable management strategy. The study of triatomine neuroendocrinology is of interest to both basic and applied entomology, motivating an active research field. This has been boosted in recent years by the publication of the *Rhodnius prolixus* genome, which allowed a comprehensive characterization of the neuropeptidergic complement, transcriptomic, proteomic and related molecular and physiological research. Even though research on triatomine species other than *R. prolixus* is scarce for neuropeptides and their receptors, several publications have emerged in recent years. This allowed performing structural and functional comparisons and proposing both conserved and species-specific characteristics for the subfamily. Here we comprehensively review the triatomine neuropeptidergic system and compare the results obtained for the different species studied to date.

Keywords Neuropeptides · G-protein coupled receptors · *Rhodnius prolixus* · *Triatoma* spp. · Hormones

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1 Introduction

Intercellular signalling appeared early in evolution; it is found in ancestral groups such as protists (Le Roith et al. 1980), cnidarians (Grimmelikhuijzen et al. 1996) and turbellarians (Adami et al. 2011) and vertebrates as well. In multicellular organisms, the cells communicate through chemical signals of different nature, including mineral ions, lipids, amino acids and peptides (Nässel and Zandawala 2019). Cellular communication can occur between cells that are nearby (nanometres) or distant (see Hartenstein 2006). In the first case, the chemical signals (neurotransmitters) are released into the extracellular space at particular sites called synapses. Among the remote communication mechanisms are the autocrine, paracrine and endocrine systems. In autocrine and paracrine systems, neuroactive chemicals are released into the extracellular space and act locally, regulating the activity of the same cells that produce the signal (autocrine) or neighbouring cells (paracrine). In the endocrine system, the hormones are released into circulating fluid, like haemolymph or blood, and spread to act on a systemic level.

In insects, neuropeptides (NPs) can act as hormones or neurotransmitters. Mature biologically active NPs are generated through the proteolytic cleavage of larger precursors. The NP precursors are synthesized, transported into the rough endoplasmic reticulum (RER) and processed in the Golgi apparatus, where the mature peptides are stored in vesicles until the moment of exocytosis (Pow and Golding 1987). The NP precursors present a hydrophobic sequence of around 20 amino acids in length in their N-terminal region, which leads them to the secretory pathway (Sossin et al. 1989). A canonical NP precursor also presents a variable number of peptides separated by dibasic or monobasic sites, where proteolytic cleavages occur (Nässel 2002; Veenstra 2000). In many cases, the peptides are enzymatically modified after cleavage by C-terminal amidation, oxidation, acetylation, among others. Post-translational modifications contribute to the stability of the peptides and are important for their interaction with receptors. Frequently, a neuropeptide precursor gives rise to multiple peptides that share a common motif that distinguishes them as a family (Nässel 2002). Peptides originated from a single precursor can have similar roles, while in other cases, they can have different physiological actions (Zhang et al. 2005). With few exceptions, neuropeptide precursors are produced in the central nervous system (CNS), but they can also be produced in peripheral tissues. In the latter case, peptide production could suggest a role related to a particular structure.

Most neuropeptides exert their physiological role by acting through G-protein coupled receptors (GPCRs), with the reported exception of those acting through guanylate cyclase receptors (eclosion hormone (EH) and neuropeptide-like precursor 1 (NPLP1)) or tyrosine kinase receptors (insulin-like peptides (ILPs), neuroparsin A (NPA) and prothoracicotropic hormone (PTTH)). The relevance of GPCRs in endocrine regulation motivated database searches and phylogenetic analysis to comprehensively identify receptors for neuropeptides in the *R. prolixus* genome and *Triatoma* spp. (Ons et al. 2015; Ons 2017). These studies allowed hypothesizing

ligands for each GPCR-like gene identified. However, to unequivocally assign a ligand to a receptor, functional deorphanization assays are required. Functional deorphanization of *R. prolixus* GPCRs was accelerated in the last years. To date, RhoprACPR (Zandawala et al. 2015a), RhoprAKHR (Zandawala et al. 2015b), RhoprFGLamideR (Zandawala and Orchard 2015), RhoprCT-DHR (Zandawala et al. 2013), RhoprMIPR (Paluzzi et al. 2014), RhoprCZR (Hamoudi et al. 2016), RhoprCCAPR (Dohee Lee et al. 2013b), RhoprCAPAR (Paluzzi et al. 2010), RhoprMSR (Lee et al. 2015), RhoprCRFR (Lee et al. 2016), RhoprNPFR (Sedra et al. 2018) and RhoprPKR (Paluzzi and O'Donnell 2012) have been functionally assigned to their respective ligands. Other putative GPCRs have been detected in the *R. prolixus* genome, and phylogenetic analyses have allowed suggesting ligands based on their similarity with previously deorphanized *D. melanogaster* GPCRs (Fig. 1). Additionally, four GPCRs detected in the *R. prolixus* genome are orthologues of orphan receptors, whose ligands have not been determined for any species (Fig. 1). In parallel, the receptors for OKA, OKB, NVP-like, IDLSRF-like and ITG-like NPs remain unknown. The identification of a neuropeptide GPCR in a particular organ or tissue suggests a physiological role for a neuropeptide system. Table 1 describes the NP precursors identified in *R. prolixus*. Table 1 also includes information about physiological experiments and expression patterns, when available. Table 2 lists NP receptor genes identified for this species, as well as expression pattern information, when available.

Figure 1 shows a phylogenetic analysis of the putative neuropeptide-related GPCRs identified in *T. infestans*, *T. dimidiata* and *T. pallidipennis* transcriptomes, together with those deorphanized in *Drosophila melanogaster* and *R. prolixus*. Sequences encoding putative neuropeptide GPCRs in the *R. prolixus* genome that are yet to be deorphanized are also included. A similar analysis was previously performed (Ons et al. 2015); however, the inclusion of recently deorphanized sequences from *R. prolixus* allows refining the phylogenetic hypotheses for the putative GPCRs of *Triatoma* spp. In this way, the putative TripaCRFR, TridiCRFR, TridiCT-DHR, TridiProctolinR, TridiCCAPR, TriinCZR, TridiPKR, TriinACPR, TripaPKR, TridiSNFR, TripaATR and TriinTKR could be identified (Fig. 1).

Because of its relevance in the control of vital processes, the neuroendocrine system of insects (neuropeptides and their receptors) is considered a potential source of targets for next-generation insecticides (Audsley and Down 2015; Verlinden et al. 2014). The use of several pesticides has been restricted or prohibited due to environmental and toxicological considerations. Moreover, insecticide resistance has been selected and expanded in most harmful species. Of particular relevance is the case of pyrethroid resistance in *T. infestans,* given its implications for the control of Chagas' disease (Capriotti et al. 2014; Fabro et al. 2012; Sierra et al. 2016). Hence, the search for targets for new-generation insecticides is becoming urgent. The integrated vector management strategy suggested by the World Health Organization (WHO 2012) requires a multiplicity of strategies, including the development of compounds with low environmental impact. It was proposed that species specificity and environmental compatibility could be achieved with exploiting neuroendocrine compounds. Given that these molecules would be designed based on

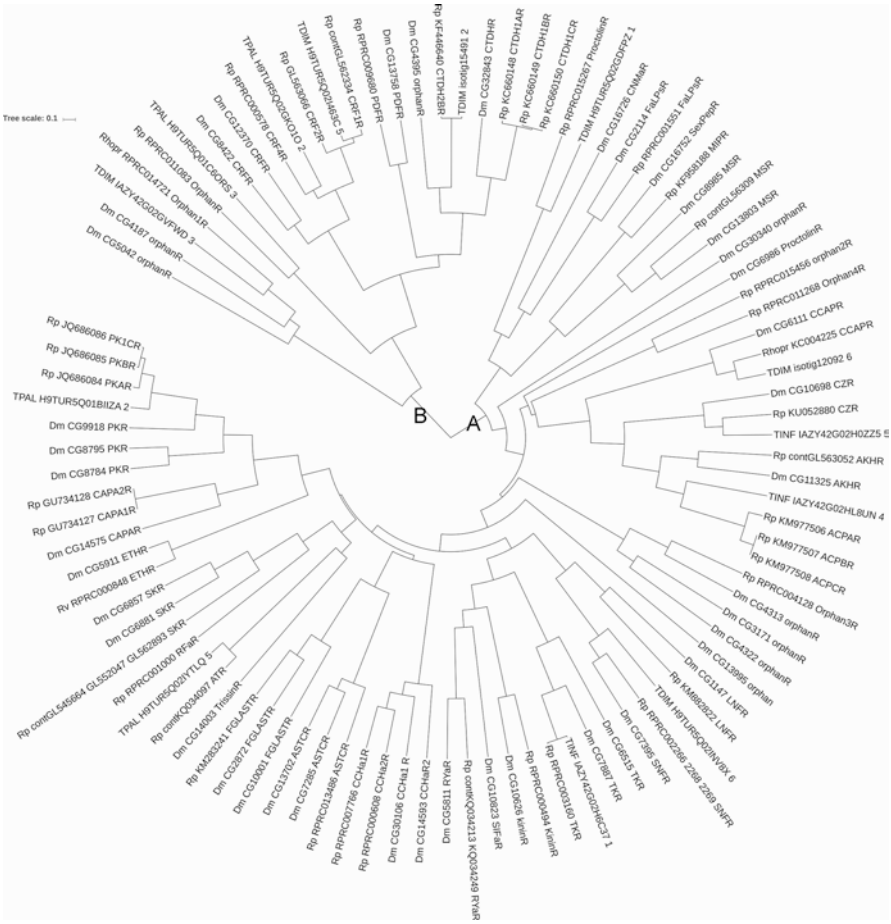

Fig. 1 Bayesian phylogenetic analysis of neuropeptide GPCRs from triatomines. The tree is based on amino acid Clustal Omega alignments (Sievers and Higgins 2018) and was performed with the software BEAST v1.8.1 (Drummond and Rambaut 2007) and Bayesian inference method in the CIPRES Science Gateway (Miller et al. 2015) with Markov chain Monte Carlo (MCMC) of two million and a burnin of 500 as parameters. Numbers on the nodes indicate posterior probability. Dm: *D. melanogaster*; Rp: *R. prolixus*; Td: *T. dimidiata;* Ti: *T. infestans*; Tp: *T. pallidipennis*. *D. melanogaster* sequences used for the construction of the tree are available in Flybase (https://flybase.org/); Genebank accession numbers when available are provided. Sequences inferred from transcriptomes are available in Ons et al. (2016)

physiological ligands, they could be less prone to induce the classic mechanisms of resistance, such as enhanced detoxification or mutations at the target site, when compared to neurotoxic control tools (Verlinden et al. 2014). Pseudopeptides and peptide mimetics interfering with insect physiology have already been assayed on disease vectors, including *R. prolixus* (Lange et al. 2015). Therefore, research on structural and functional characterization of the neuroendocrine system in triato-

Table 1 Neuropeptide and neurohormone precursors described in *Rhodnius prolixus*

Elevenin-2	RPRC003084	KQ034317
Ecdysis triggering hormone	RPRC014486/rp_asb-75167*	KQ034462
FLP (FMRFamide or FIRFamide)	RPRC014988	KQ035274
Glycoprotein hormone-2	RPRC007092	KQ034094
Glycoprotein hormone-5	ND	KQ034094
IDLSRF-like peptide	RPRC000351	KQ034112
Insulin-like peptide 2	RPRC007020/KT896507/rp_asb-36983*	KQ034142
Insulin-like peptide 3	ND	KQ034142
Insulin-like peptide 6	Rp-nr-32172*	KQ034222
Ion transport peptide isoform A	GQ253921	KQ034208
Ion transport peptide isoform B	RPRC000519/GU207866	KQ034208
ITG-like	ND	KQ034255
Kinin	RPRC000022/BK007870	KQ034106
Long Neuropeptide F	RPRC008107/KT898124	KQ034255
Myosuppressin	RPRC000203/GQ344501	KQ034384
Natalisin	RPRC003680	KQ034106
Neuroparsin	RPRC002095/GU207864	KQ034340
Neuropeptide like precursor 1	RPRC011668/GU207865/rp_asb-45918*/ comp274602#	KQ034238
NVP-like	RPRC003052	ACPB03040762
Orcokinin isoform A	RPRC014678/FJ167860	KQ034149
Orcokinin isoform B	JF761320	KQ034149
Orcokinin isoform C	KF179047	KQ034149
Pigment dispersing factor	comp36457#	KQ034061
Proctolin	RPRC000390/JN543225	KQ034188
Pyrokinin (PBAN)	GU230851	KQ034521
RYamide	RPRC000461	KQ035177
Short Neuropeptide F	GQ452380	KQ034092
SIFamide	GQ253922	KQ035590
Sulphakinins	GQ162784	KQ034228
Tachykinins	RPRC000843/GQ162785	ACPB03026326

RPRC: vectorbase ID, *: trasncripts ID in Ribeiro et al. 2014, #: trasncripts ID Latorre-Estivalis et al 2019
ND not determined

mines is of interest for both basic and applied entomology. Significant progress in this field has been achieved in the last years, especially for *R. prolixus*, the only triatomine species with a sequenced genome available (Mesquita et al. 2015), facilitating molecular and genetic studies.

Table 2 Receptors for neuropeptides described in *Rhodnius prolixus*

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
Adipokinetic hormone/ corazonin-related peptide A	RPRC000057+RPRC004783/ KM975506	KQ034104, KQ035406 and KQ034241	Yes	GCPR family A	CNS, male and female reproductive tissues
Adipokinetic hormone/ corazonin-related peptide B	RPRC000057+RPRC004783/ KM975507	KQ034104, KQ035406 and KQ034241	Yes	GCPR family A	CNS, male and female reproductive tissues
Adipokinetic hormone/ corazonin-related peptide C	RPRC000057+RPRC004783/ KM975508	KQ034104, KQ035406 and KQ034241	Yes	GCPR family A	CNS, male and female reproductive tissues
Adipokinetic hormone	AIJ49751	KQ034132	Yes	GCPR family A	Fat body, flight muscle, dorsal vessel, CNS, female reproductive tissue, antennae
Allatotropin	KF740716	KQ034097	No	GCPR family A	Anterior midgut, hindgut, dorsal vessel, MT, ovary and antennae
Allatostatin A	RPRC004705+RPRC004706	KQ034532	No	GCPR family A	CNS, dorsal vessel, anterior midgut, posterior midgut and hindgut
Allatostatin A	RPRC004708/KM283241	KQ034532	Yes	GCPR family A	CNS, dorsal vessel, anterior midgut, posterior midgut and hindgut
Myoinhibitory peptide	RPRC000605/KF958188	KQ034129	Yes	GCPR family A	CNS, salivary glands, reproductive structures in male and female, dorsal vessel, MTs, prothoracic glands (along with associated fat body), hindgut and antennae
Allatostatin C	RPRC013486	KQ034333	No	GCPR family A	CNS, antennae, dorsal vessel
Bursicon	RPRC001663	KQ034113	No	GCPR family A	Antennae

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
Calcitonin-Related diuretic hormone A	RPRC009814/KC660148	KQ035556 and KQ034793	Yes	GCPR family B	CNS, dorsal vessel and testis
Calcitonin-Related diuretic hormone B	RPRC009814/KC660149	KQ035556 and KQ034793	Yes	GCPR family B	CNS, dorsal vessel, testis and antennae
Calcitonin-Related diuretic hormone C	RPRC009814/KC660150	KQ035556 and KQ034793	Yes	GCPR family B	Antennae
Calcitonin-Related diuretic hormone	RPRC004753/AHB86571	KQ034099	Yes	GCPR family B	CNS, MT, testis
Calcitonin-Related diuretic hormone	RPRC004735	KQ034099	No	GCPR family B	Antennae
Cardioacceleratory peptide (CAPA) A	RPRC000516/ADG27752	KQ034065	Yes	GCPR family A	CNS, anterior midgut, posterior midgut, hindgut and MTs
Cardioacceleratory peptide (CAPA) B	RPRC000516/ADG27753	KQ034065	No	GCPR family A	CNS
Crustacean cardioactive peptide	RPRC001248/KC004225	KQ034056	Yes	GCPR family A	CNS, hindgut, male and female reproductive system, salivary glands, antennae
Crustacean cardioactive peptide	RPRC000969+RPRC012063	KQ034561 and KQ034059	No	GCPR family A	ND
CCHamide	RPRC007766	KQ034099	No	GCPR family A	Posterior midgut
CCHamide	RPRC000608	KQ034099	No	GCPR family A	CNS, anterior midgut, posterior midgut and MTs
CNMamide	RPRC001428	KQ034058	No	GCPR family A	Antennae

(continued)

Table 2 (continued)

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
Corticotropin-releasing factor-related like diuretic hormone A	RPRC015285	KQ034141 and KQ035235	No	GCPR family B	Antennae
Corticotropin-releasing factor-related like diuretic hormone B	RPRC000578/KU942308	KQ034325	Yes	GCPR family B	Digestive tissues, upper MTs, reproductive tissues
Corticotropin-releasing factor-related like diuretic hormone C	RPRC000578/KJ407397	KQ034325	Yes	GCPR family B	Digestive tissues, upper MTs, reproductive tissues and antennae
Corazonin A	RPRC000523/AND99324	KQ034084	Yes	GCPR family A	Antennae
Corazonin B	RPRC000523/AND99325	KQ034084	No	GCPR family A	CNS, dorsal vessel, abdominal dorsal epidermis, prothoracic glands with associated fat body, female and male reproductive tissues.
Eclosion hormone	RPRC013306	KQ034473	No	Guanylate ciclase	ND
Ecdysis triggering hormone	RPRC000848+RPRC008652	KQ034066, KQ034378 and KQ034714	No	GCPR family A	Antennae
FMRFamide	RPRC001551	KQ034140	No	GCPR family A	Antennae
GPA2/GPB5	RPRC007243	KQ034109	No	GCPR family A	Antennae
Insulin	RPRC006251	KQ034536	No	Tyrosine Kinase	CNS, antennae

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
Ion transport peptide	RPRC004793	KQ034083	No	GCPR family A	Antennae
Kinin A	RPRC00494	KQ034056	No	GCPR family A	CNS, antennae
Kinin B	RPRC008570 and RPRC008649	KQ034861 and KQ034100	No	GCPR family A	Antennae
Long neuropeptide F A	KM882822	KQ034119	Yes	GCPR family A	Antennae, CNS, anterior midgut, hindgut, and female reproductive system
Long neuropeptide F B	RPRC008894	KQ034459	No	GCPR family A	Antennae
Myosuppressin	RPRC000203/AGT02812	KQ034057	Yes	GCPR family A	CNS, midgut, hindgut, reproductive tissue
Natalisin	RPRC001687	KQ034139	No	GCPR family A	Antennae
NPA	RPRC006045	KQ034063	No	Tyrosine Kinase	Antennae
Neuropeptide like precursor 1	RPRC013388	KQ034473	No	Guanylate ciclase	Antennae
Pigment dispersing factor	RPRC009680	KQ034059	No	GCPR family B	Antennae
Parathyroid hormone	RPRC011083+RPRC011086	KQ034058	No	GCPR family B	Antennae
FaLPamide/Proctolin	RPRC015267	KQ034074	No	GCPR family A	Antennae

(continued)

Table 2 (continued)

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
PBAN A	RPRC005110/AFO73269	KQ034161	No	GCPR family A	CNS, antenna, prothoracic gland and testis
PBAN B	RPRC005110/AFO73270	KQ034161	No	GCPR family A	CNS, antenna, prothoracic gland and testis
PBAN C	RPRC005110/AFO73271	KQ034161	No	GCPR family A	CNS, antenna, prothoracic gland and testis
PBAN D	RPRC008528	KQ034938	No	GCPR family A	ND
Rfamide A	RPRC014460	KQ034100	No	GCPR family A	Antennae
RYamide B	KQ034249	KQ034249 and KQ034213	No	GCPR family A	Antennae
Short neuropeptide F	RPRC002266+RPRC002268+RPRC002269	KQ034095 and KQ035872	No	GCPR family A	Antennae
SIFamide	RPRC000835	ACPB03024746	No	GCPR family A	Antennae
Sulfakinin A	RPRC003273	KQ035199, KQ035392 and KQ034565	No	GCPR family A	ND
Sulfakinin B	RPRC012816	KQ034565	No	GCPR family A	ND
Tachykinin A	RPRC003160 and RPRC000651 (99D-like)	KQ034874 and KQ034432	No	GCPR family A	CNS, antennae
Tachykinin	RPRC008022 (86C-like)	kQ035269	No	GCPR family A	Antennae
Thyrotropin-releasing hormone	RPRC001000	KQ034696	No	GCPR family A	ND

Putative ligand	Receptor ID (vectorbase/NCBI)	Genome scaffold	Functionally deorphanized	Type	Tissue expression pattern
Orphan receptor	RPRC014721	KQ034261	No	GCPR family A	CNS, antennae
Orphan receptor	RPRC004128	KQ035493	No	GCPR family A	ND
Orphan receptor	RPRC008364	KQ034143	No	GCPR family A	CNS

ND not determined

2 Structure of the Neuroendocrine System in Triatomines

The first study isolating and characterizing a neuropeptide precursor gene in hemipterans was published in 2008 to describe the *RhoprCAPA* transcript sequence and its expression pattern (Paluzzi et al. 2008). One year later, the advent of peptidomic approaches allowed the de novo sequencing of 42 mature neuropeptides derived from 13 precursor families in extracts from *R. prolixus* brains (Ons et al. 2009). Using this peptidomic information and homology-based searches, genomic trace archives, shotgun contigs and expressed sequence tags (EST), databases of *R. prolixus* were data-mined (Ons et al. 2011). The fragments identified were used to sequence complete transcripts by rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). This combined high-throughput strategy led to the identification of 25 complete and 6 partial neuropeptide precursor sequences in *R. prolixus* before the public availability of the assembled genome. The precursors identified encoded 194 predicted mature neuropeptides; 82 of them were detected in brain extracts by nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ons et al. 2011). After this initial characterization of the *R. prolixus* neuropeptide complement, other neuropeptide precursor transcripts were identified in this species both, by cloning and sequencing (Defferrari et al. 2016; Hamoudi et al. 2016; Orchard et al. 2011; Sterkel et al. 2012; Wulff et al. 2017; Zandawala et al. 2015a, b) and by database searches in the genome or transcriptomes that became available (Hansen et al. 2011; Jiang et al. 2013; Latorre-Estivalis et al. 2020; Ons 2017). Many alternative splicing isoforms were confirmed by cloning and sequencing, whereas others were detected in assembled transcriptomes. In the latter case, errors in the assembly could exist. However, careful manual analysis of the assembled sequences, considering conserved domains and localization in neighbouring regions in the genome, should be performed for possible isoforms. This allows predicting whether a particular isoform in a transcriptome is the result of a misassembled transcript or a real splicing variant. In total, 54 neuropeptide precursors and 4 protein hormone transcripts belonging to 41 conserved families have been identified in *R. prolixus* to date (Table 1).

For triatomine species other than *R. prolixus*, information on the neuroendocrine system is scarce because a smaller number of laboratories have worked with them and there is still no genomic information. However, the mining of transcriptomic databases has allowed identifying 20 neuropeptide precursor transcripts in *Triatoma infestans*, 16 in *Triatoma dimidiata* and 13 in *Triatoma pallidipennis*. Subsequently, the presence of 59 mature neuropeptides was validated by LC-MS/MS in the brain and nervous ganglia of *T. infestans* (Marco et al. 2013; Traverso et al. 2017). Adipokinetic hormone (AKH) was also identified by LC-MS/MS in *Dipetalogaster maxima* and *Panstrongylus megistus* (Marco et al. 2013). Furthermore, putative GPCRs for neuropeptide for *Triatoma* spp. have been identified through database searches of transcriptomic sequences and phylogenetic analyses (Ons et al. 2015) (Fig. 1).

The analysis of sequences from triatomine NP precursors and mature peptides led to the identification of several particularities when compared to other insect families (Lavore et al. 2018). Comprehensive analysis of NP precursors from related species revealed that many of these rare characteristics are shared among heteropterans (Lavore et al. 2018): the presence of an unusual motif in the core peptides encoded in the FMRFamide precursor, the C-terminal sequence LGL-amide instead of the conserved FGL-amide in peptides encoded in FGLa-allatostatin (AST), the unusual C-terminal pentapeptide FSXWA-amide in the kinin precursor, the uncommon motif W(7X)W-amide in myoinhibitory peptide (MIP), the rare GYMRF-amide sequence in one of the core peptides encoded in the sulfakinin (SK) precursor, and a neuropeptide F (sNPF) precursor that is shorter than most of its orthologues (Ons et al. 2009, 2011; Traverso et al. 2016). Besides, the triatomine myosuppressin (MS) seems unique at the sequence level; it possesses an Ile in the third position instead of the usual Val and the sequence FMRF-amide in the C-terminal instead of the more conserved FLRF-amide (Traverso et al. 2016). Moreover, even though a high level of conservation generally exists in triatomine neuropeptides, the C-terminal conserved motif of the ecdysis triggering hormone (ETH) mature neuropeptide in *R. prolixus* (as in most insect species) is VPRX-amide (X = Leu or Ile) (Ons et al. 2011). However, in *T. pallidipennis*, *T. dimidiata* and *T. infestans* this motif is LPRI-amide, previously detected only in *Nilaparvata lugens* (Tanaka et al. 2014; Traverso et al. 2016). Moreover, the AKH mature peptide is an octapeptide in *R. prolixus* and *P. megistus*, a nonapeptide in *T. infestans,* and a decapeptide in *D. maxima* (Marco et al. 2013; Ons et al. 2011; Traverso et al. 2016), revealing an unusual diversity for this neuropeptide among insects from the same subfamily. From the practical point of view, the existence of different sequences is interesting since it could allow the design of neuropeptide-based insecticidal strategies that would be specific to a species or a group of species, preserving nontarget insects, including pollinators and other beneficial species.

3 Functional Studies on the Neuropeptide Systems of Triatomines

In recent years, the identification and characterization of neuropeptide precursor genes at the sequence level have facilitated research on their physiological roles. Sequence information allowed the use of techniques such as gene silencing by RNA interference (RNAi), in situ hybridization, experiments with specific synthetic peptides, development of specific antibodies, quantitative PCR, etc. Most of these studies, with a few exceptions, were performed in *R. prolixus*. A detailed review of the known roles of neuropeptides in the physiological regulation in *R. prolixus* was recently published (Ons 2017), even though new information has been incorporated since then. In this section, we summarize the literature on physiological studies with neuropeptides on *R. prolixus* and other triatomines. We include information on

isoform-specific expression patterns of NPs and their receptors (summarized in Tables 1 and 2), which could give clues to test physiological hypotheses in the future.

AKH/corazonin-related peptides (ACP): This neuropeptide system was discovered in insects in 2010 (Hansen et al. 2010). ACP is closely related to AKH and corazonin (CRZ) at the sequence level. Phylogenetic analysis suggested that ACPR and AKHR were originated in Arthropoda by duplication of the gonadotropin-releasing hormone receptor gene (Sakai et al. 2017; Tian et al. 2016). However, the physiological role of ACP has not been well established to date in any species. In *R. prolixus*, the ACP precursor gene has been cloned and sequenced (Zandawala et al. 2015a) and the presence of peptides derived from this precursor has been demonstrated through immunohistochemistry (IHQ) in cells located in the brain, but not in neurohemal sites or suboesophageal (SOG), prothoracic (PTG) and mesometathoracic (MTGM) ganglia (Patel et al. 2014). These ganglia, however, receive ACP-positive neuron projections (Patel et al. 2014). At the peripheral level, ACP seems to be expressed in the dorsal vessel, posterior midgut, hindgut and reproductive structures from both sexes (Zandawala et al. 2015a). Three different isoforms were described for the ACP GPCR. They were found to be expressed in the CNS, reproductive tissues from both sexes and antennae (Zandawala et al. 2015a; Latorre-Estivalis et al. 2020). Physiological assays have been performed to study the role of ACP in lipid mobilization and regulation of heart rate (such as AKH and CRZ respectively, see below) revealing negative results (Patel et al. 2014).

AKH: A role of AKH in lipid mobilization from the fat body to the haemolymph was demonstrated in *R. prolixus* using both in vivo injection of the synthetic AKH mature peptide (Patel et al. 2014) and RNAi-mediated gene silencing (Alves-Bezerra et al. 2015; Zandawala et al. 2015a, b). Injections of conspecific AKH in *D. maxima*, *R. prolixus* and *T. infestans* augmented the lipid levels in haemolymph (Marco et al. 2013). Even though significant, the amount of lipids mobilized to haemolymph in bioassays with triatomines is small compared to results obtained for *Orthoptera* and *Lepidoptera* spp. (Gäde 1990; Ziegler 1990). As in all the other insect species studied, the AKH gene is expressed in the *corpora cardiaca* (CC) of *R. prolixus* (Zandawala et al. 2015a, b). Interestingly, its expression was also detected in an ovary transcriptome (http://rhodnius.iq.ufrj.br/index.php?option=com_content&view=article&id=22&Itemid=34; identifier rp_asb-50392). A new splicing variant, AKH-B, was recently found to be expressed in *R. prolixus* antennae (Latorre-Estivalis et al. 2020).

The AKH GPCR was found to be more abundant in the fat body and the flight muscle, but it was also detected in the dorsal vessel, CNS, female reproductive tissue and antennae (with higher transcript levels in fifth instar nymph antennae compared to adults) (Alves-Bezerra et al. 2015; Latorre-Estivalis et al. 2020; Zandawala et al. 2015b).

Allatotropin (AT): As with most insect neuropeptides, the *AT* gene is mainly expressed in the CNS of *R. prolixus,* and its expression in antennae was recently

reported, with higher expression in males compared to females and nymphs (Latorre-Estivalis et al. 2020). Nevertheless, processes containing AT were detected in the salivary glands and hindgut (Masood and Orchard 2014). Results obtained in vitro point to a role of RhoprAT in the expulsion of saliva and a synergistic effect with serotonin (5HT) to increase the frequency of the aorta contractions (Masood and Orchard 2014; Villalobos-Sambucaro et al. 2015). A synergism with 5HT was also observed in *T. infestans* (Sterkel et al. 2010). Both the myostimulatory and cardioacceleratory effects of AT were higher in males than in females (Sterkel et al. 2010). Besides, in vivo results using injected antibodies suggested a diuretic effect for AT in *R. prolixus* and *T. infestans* (Santini and Ronderos 2007; Villalobos-Sambucaro et al. 2015). A myostimulatory action on the hindgut and the expression of AT in Malpighian tubules (MTs) was also proposed, suggesting the action of AT in regulating diuresis in an autocrine or paracrine way in *T. infestans* (Santini and Ronderos 2009). However, the expression of the AT precursor was not confirmed in an *R. prolixus* MT transcriptome (Ons 2017). The expression of the AT GPCR in the midgut, hindgut, dorsal vessel and MT (Villalobos-Sambucaro et al. 2015) reinforces the hypothesis of AT regulating diuresis-related events. Expression of the gene coding for this GPCR was also detected in the ovary transcriptome (Ons 2017), pointing to a pleiotropic effect of AT in triatomines.

Allatostatins (AST)*:* The name AST designates three different families of insect neuropeptides: FGLamide-AST (or AST-A), myoinhibitory peptide (MIP) or AST-B and PISCF-ASTs (or AST-Cs). These structurally unrelated families were originally named AST based on their ability to inhibit the production of juvenile hormone (JH) in the *corpora allata* (CA) in different insect species (Veenstra 2009). However, functions different from their allatostatic role have been described for these molecules (see below).

Rhopr-FGLa/AST gene is highly expressed in the CNS and the antennae of nymphs (Zandawala et al. 2012; Latorre-Estivalis et al. 2020). Applications of the synthetic peptide in vitro revealed a myoinhibitory effect on the anterior midgut and hindgut in *R. prolixus* and reduced heartbeat frequency (Zandawala et al. 2012; Sarkar et al. 2003). The expression of *Rhopr-FGLa/AST* receptor was found in the CNS, anterior midgut, posterior midgut, male and female reproductive structures and dorsal vessel. Bioassays performed to determine whether it affects fluid transport across the anterior midgut or the rate of secretion by MTs gave negative results (Zandawala and Orchard 2013). Considering the expression pattern of *RhoprFGLamide-ASTR*, a role in the regulation of reproduction was proposed (Zandawala and Orchard 2015).

In *R. prolixus*, MIP neuropeptides possess a myoinhibitory effect on the oviducts and hindgut (Paluzzi et al. 2015; Sedra et al. 2014). This is in agreement with the detection of processes innervating male and female reproductive structures and hindgut. Processes containing MIP were also detected in salivary glands. MIP-positive neurons are present throughout the CNS (Lange et al. 2012), and MIP transcripts were also detected in *R. prolixus* nymph antennae (Latorre-Estivalis et al. 2020).

MIP GPCR undergoes alternative splicing. The isoform A is expressed mainly in the CNS and salivary glands, but also the reproductive structures, dorsal vessel, MTs and prothoracic gland along with its associated fat body and the hindgut (Paluzzi et al. 2015). Isoform B has only been detected in an antennal transcriptome (Latorre-Estivalis et al. 2020).

According to their conserved core peptide, AST-C precursor genes were classified as AST-C (or PISCF-AST), AST double C and AST triple C (Veenstra 2016). Diptera, Coleoptera and Lepidoptera possess PISCF-AST and double C paralogues (Veenstra 2016), whereas double and triple C ASTs were reported in Hemiptera (including *R. prolixus* and *T. infestans*) (Ons et al. 2011; Traverso et al. 2016) and in most Hymenoptera (Chang et al. 2018). Remarkably, until recently AST triple Cs in hemipterans were wrongly classified as PISCF-ASTs but, according to the taxonomy proposed with the detection of three paralogues in arthropods (Veenstra 2016), they should be classified as AST triple C.

A cross-species assay using synthetic AST-C and AT from *Aedes aegypti* suggested that AeAST-CCC antagonized the synergistic myostimulatory effect of AeAT and 5HT in *R prolixus* (Villalobos-Sambucaro et al. 2016). *RhoprAST-CCC* expression has been detected in CNS (Ons et al. 2011). Remarkably, *RhoprAST-CC* and *RhproAST-CCC* are among the most highly expressed neuropeptide genes in *R. prolixus* antennae. *RhoprASTR* gene is expressed in the CNS, antennae and dorsal vessel (Ons et al. 2015; Villalobos-Sambucaro et al. 2015; Latorre-Estivalis et al. 2020).

Calcitonin-like diuretic hormone (CT-DH): CT-DH has a small diuretic effect in *R. prolixus*, revealed by a limited increase in the secretion rate by the MTs in vitro (Te Brugge et al. 2005); no effects on the secreted fluid or the rate of transport across the anterior midgut were detected (Te Brugge et al. 2009). Besides, CT-DH neuropeptides increased the frequency of contractions (Te Brugge et al. 2009) and the cyclic AMP content of the anterior midgut (Te Brugge et al. 2009); hence, a role of CT-DH in the regulation of feeding-related events was proposed (Zandawala et al. 2013). *RhoprCT-DH* is expressed in the CNS, including neurohemal sites such as the *CC* and abdominal nerves. It was also found in processes over the salivary glands and hindgut (Te Brugge et al. 2005). Three splicing variants were described for this gene (Ons et al. 2011; Zandawala et al. 2011). The expression of *RhoprCT-DH* isoform C in testes (Ons 2017) suggests a role in male reproductive physiology. Isoforms A and B were also detected in antennae (Latorre-Estivalis et al. 2020).

Two different genes that encode the putative CT-DH GPCR were identified in the *R. prolixus* genome (Zandawala et al. 2013). Three different isoforms were described for the *RhoprCT-DH1* GPCR (A, B and C). Isoform A does not possess the characteristic seven transmembrane domains; isoforms B and C differ only by two amino acids (Zandawala et al. 2013). High levels of expression of the truncated *RhoprCT-DHR1A* gene were detected in the testes, but also lower levels in the ovaries and dorsal vessel. Besides, *RhoprCT-DH1* GPCRs are expressed in the CNS, dorsal vessel, salivary glands, hindgut, testes, ovaries,

prothoracic glands and antennae (Latorre-Estivalis et al. 2020; Zandawala et al. 2013).

RhoprCT-DHR2 is expressed as isoforms A (truncated) and B (possessing all the characteristics of a GPCR). Isoform A was detected at low levels in the CNS, whereas isoform B was detected in the CNS, MTs and testes. Functional analysis demonstrated a dose-response effect of synthetic RhoprCT-DH in the activation of RhoprCT-DHR1B, RhoprCT-DHR1C and RhoprCT-DHR2B (Zandawala et al. 2013). *RhoprCT-DHR3* is highly expressed in the antennae of *R. prolixus*, particularly in the case of male and female antennae (Latorre-Estivalis et al. 2020).

CAPA: RhoprCAPA precursor encodes both CAPA and pyrokinin mature peptides. The CAPA-type peptides inhibited fluid transport across the anterior midgut and secretion by MTs stimulated with 5HT (Ianowski et al. 2010). Indeed, the CAPA neuropeptide is the most potent antidiuretic factor reported to date in *R. prolixus* in vitro. However, the secretion rate of MTs stimulated with the diuretic peptide CRF (see below) was not modified by CAPA peptides, but abolished the synergism that occurs between 5HT and RhoprCRF (Ianowski et al. 2010). To date, no in vivo studies have been performed to confirm the antidiuretic effect of CAPA in triatomines.

CCHamide: Two CCHamide encoding genes are present in the *R. prolixus* genome, RhoprCCHamide 1 and RhoprCCHamide 2 (Hansen et al. 2011). These genes differ widely at the sequence level, even in the core peptides encoded. Three isoforms were detected for the RhoprCCHamide 2 gene: isoform A expressed in the CNS (Ons et al. 2011), isoform B in anterior midgut and testis transcriptomes (Ons 2017), while isoform C expression was recently detected in antennae (Latorre-Estivalis et al. 2020). Expression of the RhoprCCHamide2 gene was also detected in MTs by QPCR (Capriotti et al. 2019).

Two *CCHamide GPCRs* are present in the *R. prolixus* genome (Ons et al. 2016): RPRC007766 is expressed in the posterior midgut, whereas expression of RPRC000608 was detected in the anterior and posterior midgut, and the MTs (Capriotti et al. 2019).

CCHamide2 has been recently reported to be involved in the regulation of diuresis based on results obtained both in vitro and in vivo (Capriotti et al. 2019). The involvement of CCHamide2 in diuresis-related events is reinforced by its expression pattern in the CNS, MTs and anterior midgut. The expression of its two GPCRs in the anterior and posterior midgut, as well as the MTs also reinforces this idea (Capriotti et al. 2019). Interestingly, even though RNAi silencing of the precursor indicates a net antidiuretic effect for RhoprCCHamide2, the in vitro bioassays point to an opposite activity on the MTs and anterior midgut. A synthetic peptide identical to the core peptide encoded in this precursor enhanced the 5HT-induced secretion by MTs and inhibited 5HT-induced absorption across the anterior midgut (Capriotti et al. 2019). The case of RhoprCCHamide2 is remarkable, given that no other neuropeptide presented opposite effects on the anterior midgut and MTs. It seems to

reflect the importance of a well-tuned diuretic process in triatomine insects during different moments after the blood meal.

Corazonin (CRZ): The only published effect of CRZ in triatomines is a strong stimulation of the heartbeat frequency in vitro (Patel et al. 2014). Even though CRZ initiates the ecdysis process in different insect species (Zitňan and Adams 2012), silencing of the *CRZ GPCR* gene did not affect the ecdysis of fourth instar *R. prolixus* nymphs (Hamoudi et al. 2016). Immunohistochemical analysis of CRZ presence was performed in *R. prolixus* and *T. infestans* (Patel et al. 2014; Settembrini et al. 2011), revealing similar expression patterns in the CNS of both triatomines. RhoprCRZ was also detected in the haemolymph by LC-MS/MS, indicating that its release from the CNS seems to exert a hormonal role (Ons et al. 2011). Transcripts for this gene were also detected in a transcriptome from testes (Ons 2017).

R. prolixus genome encodes one *CRZ GPCR* that presents two splicing variants. The expression of one isoform was detected in the CNS, dorsal vessel, abdominal dorsal epidermis, prothoracic glands with the associated fat body, and reproductive tissues from both sexes (Hamoudi et al. 2016), while a second isoform was detected in antennae (Latorre-Estivalis et al. 2020).

CRF-DH: CRF-like DH is the strongest diuretic neuropeptide identified in *R. prolixus* to date. Its potent diuretic activity has been demonstrated in vitro; RhoprCRF stimulates both, rate of absorption through the anterior midgut and the secretion rate by the MTs and the (Te Brugge et al. 2009, 2011). RhoprCRF-like DH expression was detected throughout the CNS and in antennae (Te Brugge et al. 2009, 2011; Latorre-Estivalis et al. 2020). The presence of CRF-DH peptides in the haemolymph in response to blood-gorging was suggested (Lee et al. 2016). Recent results demonstrated that the injection of synthetic RhoprCRF peptide provoked a significant reduction in blood intake both in adult and fifth instar nymphs and in the number of eggs produced and laid by mated females (Mollayeva et al. 2018). These results indicate a pleiotropic role of RhoprCRF, stimulating diuresis and inhibiting feeding and reproduction.

The *R. prolixus* genome contains four paralogue genes encoding *CRF-like GPCRs* (Ons et al. 2016), even though only two were predicted in the automatic prediction of the genome, named *RhproCRF-DHR1* and *RhproCRF-DHR2* (Lee et al. 2016; Ons et al. 2016). The latter has been cloned, sequenced and functionally deorphanized (Lee et al. 2016). The expression of *RhproCRF-DHR2* was detected in digestive tissues, upper MTs and reproductive structures (Lee et al. 2016), while transcripts coding for both *RhproCRF-DH1* and *RhproCRF-DHR2* were detected in an antennae transcriptome reporting higher expression in male antennae (Latorre-Estivalis et al. 2020).

Crustacean cardioactive peptide (CCAP): CCAP neuropeptides are involved in the regulation of ecdysis in holometabolous insects (Zitňan and Adams 2012). This role seems to be conserved in Hemimetabola since *R. prolixus* nymphs that

express reduced levels of this gene present defects in ecdysis (Lee et al. 2013a). Besides, in vitro bioassays demonstrated a myostimulatory activity in the hindgut and a cardioacceleratory role for RhoprCCAP (Lee and Lange 2011). Three isoforms for this gene were described in *R. prolixus*: isoform A was detected in the CNS and antennae, isoform B in the CNS (Lee and Lange 2011; Ons et al. 2011; Latorre-Estivalis et al. 2020) and isoform C in a transcriptome from testes (Ons 2017). The three isoforms code for the same peptide precursor but they differ in the untranslated region. CCAP GPCR was cloned and sequenced in *R. prolixus*, and its involvement in the regulation of cardiac frequency was demonstrated in vitro (Lee et al. 2013b). Its expression was found in the CNS, hindgut, salivary glands, reproductive tissues from both sexes and antennae (Lee et al. 2013b; Latorre-Estivalis et al. 2020).

FMRFamide-like peptides: Results obtained using in vitro bioassays demonstrated a role of RhoprFMRFs in ovulation and oviposition, given that they stimulated contractions in ovarioles, ovaries, oviducts and bursa (Sedra and Lange 2014). The detection of RhoprFMRFamide in the CNS and projections reaching female reproductive structures reinforces the hypothesis of a role in female reproduction (Sedra and Lange 2014). Besides, peptides derived from the RhoprFMRFamide precursor significantly augmented the tension of hindgut muscle contraction (Al-Alkawi et al. 2017).

Kinin: The expression of Rhoprkinin seems to be regulated during postprandial diuresis, as peptides encoded in the Rhoprkinin precursor drop 1.5 and 2.5 h after feeding and augment in the CNS 4 h later, at the end of diuresis (Sterkel et al. 2011). Kinins affect the frequency of hindgut contractions and decrease the resistance and transepithelial voltage of the anterior midgut (Bhatt et al. 2014; Te Brugge et al. 2009). These results point to the involvement of Rhoprkinin in feeding-related events. Unlike in other species, kinins do not have diuretic effects in vitro in *R. prolixus* (Donini et al. 2008; Te Brugge et al. 2009; Te Brugge and Orchard 2002). Two paralogous genes coding for kinin GPCRs were found in the *R. prolixus* genome (Ons et al. 2015) and transcripts coding for these receptors were detected in antennae (Latorre-Estivalis et al. 2020).

Insulin-like peptides (ILPs): A role of RhoprILP in circadian rhythm regulation was proposed since the application of synthetic ILP in vitro reduced the expression of the clock gene *period* (Vafopoulou and Steel 2014). Besides, *RhoprILP* gene silencing provoked alterations in the distribution of lipids and carbohydrates, augmenting their levels in the haemolymph of fifth instar larvae. Based on these results, a conserved role of ILPs in the regulation of energy storage and mobilization was suggested (Defferrari et al. 2016). Three paralogous genes encoding for ILP precursors were described in the *R. prolixus* genome and named, according to their orthologues in *D. melanogaster,* as *RhoprILP2, RhoprILP3* and *RhoprILP6*. Each of these genes presents a particular tissue expression pattern: *RhoprILP-2* is expressed in the CNS, posterior midgut, fat body, ovary and antennae; *RhoprILP-3* expression was only detected in antennae of nymphs; and *RhoprILP-6* was detected in CNS, female antennae, testes, and the anterior and posterior midgut (Defferrari et al. 2016; Latorre-Estivalis et al. 2020; Ons et al.

2011; Ons 2017). *RhoprILP2* and *RhoprILP3* are encoded in neighbouring regions in the genome (Table 2), suggesting a gene duplication. One tyrosine kinase receptor for ILP was found in the genome of this species, its expression detected mainly in the CNS but at low levels also in other structures (Defferrari et al. 2018).

ITG-like peptide, NVP-like peptide and neuropeptide-like precursor 1 (NPLP1): All these precursors are conserved in most insect genomes, but their physiological roles have not been elucidated yet. ITG-like and NVP-like neuropeptide precursors are both expressed in the CNS and antennae (Latorre-Estivalis et al. 2020; Ons et al. 2011). The receptors for these neuropeptides have never been described for any species. For the *RhoprNPLP1 precursor* gene, two different isoforms were detected, each presenting a distinct expression pattern: isoform A was expressed in the CNS, ovary, testes and antennae, while isoform B was only detected in antennae (Latorre-Estivalis et al. 2020; Ons 2017; Ons et al. 2011). Mature peptides from NPLP-1 precursors were also detected in salivary glands (Sterkel et al. 2011). A putative guanylate cyclase receptor for peptides encoded in the NPLP-1 precursor could be predicted in *R. prolixus* genome (Ons 2017; Overend et al. 2012).

In the CNS of adult *R. prolixus*, the concentration of ITG-like, NVP-like and NPLP1 peptides is modulated after feeding. The concentration of peptides encoded in RhoprNVP-like, RhoprITG-like and RhoprNPLP1 precursors was decreased 24 h post-blood meal (Sterkel et al. 2011). Interestingly, the expression of both TriinNVP-like and TriinITG-like was significantly higher in a *T. infestans* high pyrethroid-resistant population compared to a susceptible strain (Traverso et al. 2016). These correlations raise hypotheses for discovering the role of these peptides in the understudied neuropeptidergic systems of insects.

Long neuropeptide F (NPF): To date, only in vitro bioassays using nonspecific synthetic peptides were reported for NPF in triatomines (Gonzalez and Orchard 2009). These assays demonstrated a myoinhibitory effect of both *DromeNPF* and *AnogaNPF* neuropeptides on *R. prolixus* fifth instar hindguts. Besides, the influx of K+ into the hindgut was not altered by the addition of DromeNPF or AnogaNPF (Gonzalez and Orchard 2009). A role in the regulation of egg production was also suggested (Sedra et al. 2018; Sedra and Lange 2016).

Transcripts coding for NPF were detected in the CNS, male antennae, fifth instar hindgut muscle fibres and lateral oviducts of adult females (Gonzalez and Orchard 2009; Latorre-Estivalis et al. 2020; Ons et al. 2011; Sedra and Lange 2016). Two paralogous genes coding for NPF GPCRs were found in the *R. prolixus* genome (Ons et al. 2016). One of them is expressed in antennae, CNS, anterior midgut, hindgut and female reproductive system (Sedra et al. 2018), while the second was only detected in antennae (Latorre-Estivalis et al. 2020).

Myosuppressin (MS): In vitro, RhoprMS inhibited the heart rate (Leander et al. 2015; Lee et al. 2012) and decreased the amplitude and frequency of spontaneous contractions of the anterior midgut and hindgut (Lee et al. 2012). The basal

tonus was increased by synthetic RhoprMS in *R. prolixus* oviducts, but no effect was detected on the amplitude of oviduct contractions (Sedra et al. 2014). This neuropeptide is expressed in the CNS and posterior midgut, whereas a pyroglutamic-modified mature peptide was found in haemolymph by nano-LC-MS/MS (Ons et al. 2011). The expression of its receptor was detected in the midgut, hindgut and fifth instar nymph reproductive organs (Lee et al. 2015).

Orcokinin (OK): In insects, two families of peptides encoded by the OK gene, named OKA and OKB, are generated by alternative splicing. OKA was first detected in the cockroach *B. germanica* (Pascual et al. 2004), whereas OKB-type neuropeptides were first reported in *R. prolixus* (Sterkel et al. 2012). Both OKA and OKB NP families were detected in all the insect genomes published to date. Furthermore, *R. prolixus* has a third splicing variant of the gene (RhoprOKC), which encodes OKB-type mature neuropeptides (Wulff et al. 2017). *RhoprOKA* expression is essential for successful ecdysis both in fourth and fifth instar nymph ecdysis. Insects in which *RhoprOKA* expression was silenced by RNAi presented a lethal phenotype during the expected ecdysis period (Wulff et al. 2017). In agreement, RhoprOKA directly or indirectly regulates the expression of genes that form the peptidergic network controlling ecdysis such as ETH, EH and CCAP (Wulff et al. 2018). Expression of *RhoprOKA* was detected in the CNS, processes reaching reproductive structures, ovary, testes and antennae (Latorre-Estivalis et al. 2020; Wulff et al. 2017). Two different OKA mature peptides were also detected in haemolymph by nano-LC-MS/MS (Ons et al. 2011).

A synthetic OKB/C peptide stimulated anterior midgut contractions in vitro dose-dependently (Wulff et al. 2018). Expression of RhoprOKB/C peptides was detected in the CNS, anterior and posterior midgut, ovary, testes, and also in processes reaching reproductive structures (Wulff et al. 2017). Semiquantitative immunohistochemistry indicated that they are released from the anterior midgut 1 h after feeding. Twenty-four hours after a blood meal, the levels of these peptides were undetectable in this tissue (Wulff et al. 2018). Based on these results, it was proposed that the different transcripts encoded by the *RhoprOK* gene could integrate signalling information to coordinate the nutritional state with development and ecdysis. These two processes are intimately coordinated in insects, particularly in triatomines. Furthermore, expression in reproductive structures suggests a role in reproduction that remains to be investigated. The finding of a role of OK in vitellogenesis in the cockroach *B. germanica* (Ons et al. 2015) reinforces the hypothesis of a conserved involvement of OKs in reproduction. The receptors for OKA and OKB peptides have not been identified in any species to date.

Pigment dispersing factor (PDF): PDF has not been extensively studied in triatomines. According to PDF-like immunoreactivity in lateral neurosecretory cells from the brain, a role in the regulation of circadian rhythms was proposed (Vafopoulou and Steel 2014). This was reinforced by semiquantitative results indicating that PDF-like immunoreactivity in *R. prolixus* male fifth instar nymph brains was abolished after 3 weeks in constant light. Once these brains were excised from the animals and transferred to darkness for 4 h, PDF-like immuno-

reactivity was restored (Vafopoulou and Steel 2014). PDF-like immunoreactivity was also induced in excised brains by incubation with bombyxin (an orthologue of insulin-like peptide from *Bombyx mori*) and BommoPTTH synthetic neuropeptides (Vafopoulou and Steel 2014). Transcripts coding for RhoprPDF and RhoprPDFR were detected in a *R. prolixus* antennal transcriptome (Latorre-Estivalis et al. 2020).

Proctolin: As for other neuropeptides, a role in stimulating the contractions of the anterior midgut, hindgut and heart in vitro was described for RhoprProctolin (Orchard et al. 2011). Besides CNS, processes presenting proctolin-like immunoreactivity were detected reaching salivary glands, hindgut, heart, alary muscles, female reproductive structures (lateral and common oviduct, spermatheca, bursa), and male reproductive structures (seminal vesicles and vas deferens). Cells expressing proctolin were also detected in the common oviduct and bursa of *R. prolixus* (Orchard et al. 2011).

Sulfakinin (SK): The SK expression pattern and physiological effects were recently studied in *R. prolixus* (Al-Alkawi et al. 2017). The gene is mainly expressed in the brain, and immunoreactive projections are extended throughout the CNS, anterior midgut and hindgut. Regarding physiological experiments, peptides encoded in RhoprSK precursor significantly augment the tension of hindgut contraction but did not have effects on the rate of aorta contractions (Al-Alkawi et al. 2017). Injection of the synthetic peptide reduced the amount of blood ingested by fifth instar *R. prolixus* nymphs, suggesting an effect on feeding behaviour (Al-Alkawi et al. 2017).

Tachykinin (TK): In *R. prolixus*, *TK* is expressed in several tissues: CNS, antennae, salivary glands, dorsal vessel, fat body and anterior and posterior midgut, and hindgut (Haddad et al. 2018; Latorre-Estivalis et al. 2020; Ons et al. 2011), suggesting a pleiotropic action. Synthetic TK induced contractions of the hindgut and increased the frequency and amplitude of contraction of salivary gland muscles in vitro (Haddad et al. 2018). Two *TK GPCRs* were found in the *R. prolixus* genome. The expression of one of them was detected in the CNS and antennae, while the other gene was only expressed in antennae (Latorre-Estivalis et al. 2020; Ons et al. 2016).

Neuropeptide families lacking physiological information in triatomines: Many neuropeptide families identified in *Triatoma* spp. transcriptomes and the *R. prolixus* genome remain to be studied by physiological approaches. Several of these systems have been well studied in other insect species, demonstrating roles in the regulation of post-embryonic development, (EH, ETH); (Zitňan and Adams 2012), sexual behaviour (SIFamide, Natalisin or MIP); (Jang et al. 2017; Jiang et al. 2013; Terhzaz et al. 2007), diuresis (neuroparsin (NPAs) and ion transport peptide) (Audsley et al. 1992; Girardie et al. 1998). Besides, other neuropeptide families, such as CCHamide 1, CNMamide, elevenin, IDLSRF-like and RYamide, belong to poorly studied neuropeptidergic systems.

In *R. prolixus*, the expression of *EH* was detected in CNS (Ons et al. 2011) and transcripts coding for its guanylate cyclase receptor were detected in antennae

(Latorre-Estivalis et al. 2020; Ons et al. 2011). SIFamide was detected in the CNS and antennae of adult insects (Ons et al. 2011. Latorre-Estivalis et al. 2020). Transcripts coding for Natalisin and its receptor were also found in antennae (Latorre-Estivalis et al. 2020). NPA was detected in the CNS, anterior midgut and antennae (Latorre-Estivalis et al. 2020; Ons et al. 2011), while its tyrosine kinase receptor was only detected in antennae (Latorre-Estivalis et al. 2020). Two different splicing forms for ITP were detected in the *R. prolixus* CNS (Ons et al. 2011), and two putative genes coding for ITP receptors were detected in the *R. prolixus* genome: RPRC008022 and RPRC004793 (Ons 2017). The expression of the NP and receptor genes was detected in antennae (Latorre-Estivalis et al. 2020). The advantages of triatomines, particularly *R. prolixus*, serving as a model species for insect physiology (Ons 2017), will allow expanding the knowledge about the physiological functions of these neuroendocrine systems.

Neuropeptide families that seem to be absent in triatomines: A small number of insect neuropeptide families could not be detected by careful homology-based searches in any of the triatomine databases generated to date, suggesting that they may be absent in the triatomine subfamily. These absent neuropeptides are inotocin, NPLPs 2–4, sex peptide, trissin and PTTH. All of them belong to the 'variable set' of neuropeptides, according to the classification proposed by Hauser et al. (2010), that is, those that are not present in all the insect genomes, different to the 'basal set' whose members are detected in all the genomes sequenced to date. However, it is still possible that homology-based searches did not detect divergent orthologous sequences. This could be the case of PTTH since its presence in *R. prolixus* was suggested by cross-species immunoreactivity assays (see Vafopoulou and Steel 2014; Vafopoulou et al. 2007).

4 Concluding Remarks

Given the genetic tools available in model species such as *D. melanogaster* and *Tribolium castaneum*, neuroendocrine research in Hemimetabola has been relegated with respect to Holometabola. Hence, research in *R. prolixus*, the second hemimetabolous species whose genome has been sequenced (Mesquita et al. 2015), constitutes an important contribution to entomology, boosting the research in Hemimetabola, and in triatomines in particular. The availability of genomic information, transcriptomic databases, sequence confirmation by gene-cloning and proteomic studies contributed to a comprehensive characterization of the neuropeptide complement. Sequence information allowed performing bioassays and GPCR deorphanization by using species-specific peptides. As a model in entomological research, *R. prolixus* presents advantages for the study of diuresis (given the size and structure of its MTs), hematophagy and post-embryological development given that ecdysis occurs a fixed number of days after feeding.

The availability of several organ-specific transcriptomes and RT-PCR studies in *R. prolixus* revealed that neuropeptides are frequently expressed in the CNS as well as in structures that are not usually considered as endocrine. In particular, a global observation of the results obtained to date indicates that components of many neuropeptidergic systems are expressed in testes and antennae. The former suggests an important local endocrine regulation in these structures. Moreover, several splicing variants seem to be organ-specific, for both neuropeptide gene precursors and GPCRs. This may suggest different roles for alternative isoforms. However, differences originating due to diverse assembly strategies and depth of the various transcriptomes analysed cannot be ruled out.

Even though a high degree of conservation in the neuroendocrine system is expected among insects belonging to the same family, particularities observed for triatomine neuropeptides from different species indicate that this is not always the case. A recent paper comparing the neuropeptide complement in genomic or transcriptomic databases of species of Coleoptera spp. revealed that neuropeptidomes of species belonging to the same order could usually present significant differences for certain gene families (Veenstra 2019). The observations in triatomines indicate that although most characteristics are conserved in closely related species, differences also exist at the family level. Hence, studies on the structure and physiology of the neuroendocrine system of triatomine species other than *R. prolixus* will be important for the study of triatomine neuroendocrinology. The availability of high-throughput techniques and the publication of the *T. infestans* genome, besides the information already collected for *R. prolixus,* will allow boosting this field. The importance of this research, besides its significance for molecular, comparative and phylogenetic knowledge, is on expanding the knowledge on possible targets for developing next-generation insecticides against *T. cruzi* vectors.

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Sensory Biology of Triatomines

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Abstract Sensory systems mediate behaviors such as food, mate and shelter search, habitat selection, avoidance of environmental risks, escape from predators, and communication with conspecifics. Yet, immersed in an environment full of flowing information, triatomines, as other living organisms, must recognize and make use of adequate and sparse sensory stimuli. Specialized sensory structures allow the detection of a great variety of stimuli, such as visual cues, water vapor, heat, infrared radiation, vibratory signals, and a plethora of volatile and nonvolatile chemicals. Thus, different and specialized sensory capacities allow these animals an efficient exploitation of their environments according to their evolutionary history in specific ecotopes and particular selective pressures.

Keywords Olfaction · Taste · Thermal sense · Vision · Mechanoreception

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1 Introduction

Vision, olfaction, gustation, and mechano, thermo, and hygric reception are crucial sensory systems supplying the nervous system of animals with fundamental information. In its turn, the proper integration of such complex arrays of cues[1] and signals[2] allows individuals to make appropriate decisions according to the particular requirements of their biology. Indeed, the identification of an adequate habitat offering optimal environmental conditions in which to develop, copulate, and lay eggs is essential for triatomine survival. The visual system of triatomine bugs, for example, is well adapted to detect changes in light intensity. Still, it is also capable of mediating rapid escape responses and attraction to light sources from human dwellings, as triatomines probably disperse by flight. Finally, light perception allows triatomines to temporally synchronize their internal clocks, as many activities of these bugs are regulated by a circadian system. They rely on their thermal sense to acquire information about ambient temperature and, based on it, adjust their preferences to choose or abandon a particular habitat. As a result, triatomines assure their optimal development and physiological performance under adequate temperature conditions. The thermopreference of triatomines is not the only dominant factor for habitat selection; they also express a marked xeropreference when choosing resting sites, that is, selecting drier narrow places. Notably, they exhibit astonishing heat sensitivity and the capacity of perceiving infrared (IR) radiation. Heat perception serves triatomines to recognize and approach warm-blooded hosts. Water vapor additionally drives the final approach toward a vertebrate host; however, it is nowadays considered that only stimulation by heat triggers proboscis extension. The chemical senses, smell and taste, give kissing bugs information about compounds present in their surroundings. Both senses can promote acceptance or repulsion upon stimulation. Olfaction allows triatomines to detect volatiles at a distance in multiple behavioral contexts, whereas the taste sense requires direct contact with the relevant chemical. Olfaction enables triatomines to find a sexual partner, to locate a host, to follow chemical landmarks signaling a refuge, but also to elicit an escape response upon detection of alarm signals emitted by conspecifics. The taste sense based on contact chemoreception becomes relevant for triatomines while they assess food quality. Likewise, it mediates footprint recognition, reinforcing bugs assembling in shelters. Finally, contact chemoreception mediates mating partner recognition. Mechanosensation complements chemical senses with valuable additional information, that is, stimulus direction and position. The numerous mechanoreceptors triatomines have on their body are probably responsible for their sensitivity to weak air movements. Furthermore, it is probable that these sensory structures also play a role in substrate recognition in the context of shelter choice. Triatomines

[1] Cues are perceptible attributes of the environment or other organisms that animals are able to detect.

[2] Signals are perceptible attributes of the environment or other organisms that had been modeled by natural selection to play a role in animal communication.

are able to produce vibrational signals, that is, to stridulate; these vibratory signals have a role in sexual communication and defense-related contexts. The stridulatory signals produced are propagated as waves through the insect body and substrate. Triatomines lack evident hearing organs, thus chordotonal organs are the putative structures involved in the detection of these signals.

In this chapter, we summarize the current knowledge about the visual, chemical, and thermal/hygric senses of triatomines with emphasis on their morphological, physiological, and molecular aspects. Less studied senses, such as mechanoreception, are also briefly presented.

2 The Visual System

Vision in triatomines is morphologically and physiologically well adapted to a crepuscular lifestyle. These insects spend daytime hours hidden in dark refuges and forage for a blood meal mostly during early nighttime. Triatomines have both positive- and negative-oriented responses to light (Ward and Finlayson 1982; Reisenman et al. 1998; Minoli and Lazzari 2006) and this might depend on factors such as the locomotion context involved (i.e., flying vs. walking) and the stimulus pattern (i.e., a punctual light source such as a house light vs. a fully illuminated environment), although these possibilities remain to be investigated (Lazzari et al. 2013). It is considered that attraction to house lights allows females to disperse and colonize new habitats (Vazquez-Prokopec et al. 2004; Jácome-Pinilla et al. 2015). Besides, triatomines respond to contrasting moving objects by turning away from them with an escape-like response, but keeping these objects in their lateral field of view (Lazzari and Varjú 1990). This so-called lateral fixation response is mediated by the dorsal part of the compound eyes and provides a mechanism by which insects can keep their host/predator visible for either approach or rapid escape (Lazzari and Varjú 1990).

2.1 The Compound Eyes

The compound eyes are the main visual organs of triatomines and are composed of many ommatidia (Insausti and Lazzari 2002). As in most insects, each ommatidium is composed of a cornea and crystalline cone, primary and secondary pigment cells, and eight photoreceptors or retinula cells. Each ommatidium is isolated from its neighbor by the pigment cells (Chapman 1998). The microvilli of the retinula cells form a rhabdom where the visual pigments concentrate and absorb the photons (Chapman 1998). Triatomines have apposition compound eyes with unfused rhabdom (Muller 1970; Reisenman et al. 2002). They exhibit a ring of six rhabdomeres formed by retinula cells 1–6, which is surrounded by a central pair of rhabdomeres formed by retinula cells 8–9 (Muller 1970; Reisenman et al. 2002). As a

consequence of a cyclic pigment migration process, light only reaches the central pair of rhabdomeres under daylight, while at night pigments disperse allowing light to reach all the rhabdomeres. The axons of the retinula cells pass the basal lamina and reach the large optic lobes in the brain (Insausti 1994).

2.2 The Ocelli

Two well-developed ocelli that develop throughout larval life (Insausti and Lazzari 2000a, b) can be found dorsolaterally, behind the compound eyes of adults. The ocelli have large lenses of about 455 μm in diameter (Insausti and Lazzari 2002). The ocellar corneagen and photoreceptor cells form a cup-like structure underneath the cuticular lenses (Insausti and Lazzari 1996, 2002). The focal plane of the ocelli lies behind the retina and, consequently, they cannot form focused images (Insausti and Lazzari 2002). The axons of photoreceptors extend into the ocellar neuropile connecting with a few thick interneurons, which terminate in thoracic centers (Insausti and Lazzari 1996, 2002).

2.3 Sensory Aspects of Vision

Visual sensitivity can be adjusted in advance to the changes in environmental light conditions, like sunrise and sunset because there is an endogenous oscillator controlling the pigment movement of compound eyes (Reisenman et al. 2002). The spectral sensitivity of triatomines ranges from wavelengths near UV (ca. 357 nm) to far-red (665–695) (Insausti et al. 2013). The screening pigments of compound eyes are of two kinds, a dark one (ommin) and a red one (xanthommatin); both are found in primary pigment cells, while only the red pigment is found in retinula cells (Insausti et al. 2013). The opsins are a large family of proteins related to the visual system of insects (Arikawa and Stavenga 2014). In the genome of *Rhodnius prolixus,* four opsins have been found (Mesquita et al. 2015), three of which were identified as orthologs of ultraviolet, green, and blue photoreceptors in other insects. Taken together, behavioral and molecular evidence supports the broad spectral sensitivity of triatomines.

On the other hand, it is likely that the ocelli, as observed in other insects, mediate fast responses related to orientation during walking and flying (Lazzari et al. 2011). While triatomine ocelli can also adapt to changes in environmental light conditions by longitudinal pigment migration within photoreceptor cells, this adaptation is not under endogenous control but is the result of a direct response to light (Lazzari et al. 2011).

3 The Olfactory Sense

Odor detection in triatomines is concentrated in the antennae and mediates host- and mate-seeking, aggregation, alarm, and escape responses (Barrozo et al. 2017 and refs. therein). Even though the same compound can be relevant in different behavioral scenarios, some substances signal specific contexts and trigger different behaviors. For example, CO_2, L-lactic acid, 1-octen-3-ol, aliphatic aldehydes (C7-C9), furan, pyridine, ammonia, and short-chain fatty acids were commonly related to host recognition (Barrozo and Lazzari 2004a, b; Mayer 1968; Taneja and Guerin 1997; Guerenstein and Guerin 2001; Diehl et al. 2003; Bernard 1974). Volatiles related with sexual communication include 3-pentanone, 2-pentanol, 2-methyl-1-butanol, other short-chain aliphatic ketones and alcohols, and dioxolanes. These volatiles are produced and released by the metasternal glands of mating pairs and have been identified as relevant sex-pheromone components of different triatomine species (Manrique et al. 2006; Pontes et al. 2008; Vitta et al. 2009; Unelius et al. 2010; Bohman et al. 2011, 2018. May-Concha 2015). Feces of several triatomines have been shown to recruit and aggregate triatomines around filter papers impregnated with them. Furthermore, it has been shown that *Triatoma infestans* use their feces to mark hiding places (Lorenzo and Lazzari 1996). Reports have divergent claims indicating that feces emit low quantities of quinazolines, acetophenone, ammonia, aliphatic amines, short-chain fatty acids, 2,3-butanediol, and acetamide (Taneja and Guerin 1997; Cruz-López et al. 1993, 1995; Lorenzo Figueiras and Lazzari 1998a, 2002; Mota et al. 2014). Mota and collaborators (2014) formulated a synthetic blend based on volatiles detected in fecal headspace that successfully induced shelter choice in bugs of three species. Triatomines also respond to warning or alarm compounds produced by disturbed adult conspecifics. Thus, the detection of danger triggers the emission of volatiles emitted by Brindley glands. Isobutyric acid is the main component emitted from these glands, although other fatty acids and esters are known to be released together, for example, 2-methyl-butyric acid (Pattenden and Staddon 1972; Kälin and Barrett 1975; Ward 1981; Manrique et al. 2006). All relevant odors mentioned are detected by neurons housed in the olfactory sensilla distributed on the antennae of triatomines.

3.1 The Antennae

The antenna of triatomines is composed of three sections: the scape, pedicellum, and flagellum, the latter being subdivided into two flagellomeres. Odors enter multiporous olfactory sensilla, and once inside the sensillar cavity, volatiles reach the dendrites of olfactory sensory neurons (OSNs). Many olfactory proteins mediate the odor detection process at the molecular level, among them odor binding proteins (OBPs), chemosensory proteins (CSPs), odor degrading enzymes (ODEs), olfactory

receptors (ORs), ionotropic receptors (IRs), and sensory neuron membrane proteins (SNMPs).

Olfactory sensilla are only found on the flagellum of *R. prolixus* but can also be found on the pedicellum of *T. infestans* (Chaĭka 1980; Catalá and Schofield 1994; Catalá 1997; Gracco and Catalá 2000; Catalá and Dujardin 2001). Three types of olfactory sensilla have been described in triatomines: basiconic, trichoid, and grooved-peg sensilla.

Basiconic sensilla are short nonarticulated structures about 25–30 μm long, which have also been called thin-walled wall-pore trichoid sensilla. Basiconic sensilla house about 21–41 OSNs in *T. infestans* and 15 OSNs in *R. prolixus* (Wigglesworth and Gillett 1934; Guerenstein and Guerin 2001). At least four OSNs were characterized in *T. infestans*, named OSN1–4 (for details see Barrozo et al. 2017). Thus, OSN1 responds to aldehydes such as heptanal, octanal, nonanal, OSN2 is stimulated with (+)-pinene and (−)-limonene, while OSN3 and OSN4 respond to terpinen-4-ol and pyridine and furan, respectively (Mayer 1968; Guerenstein and Guerin 2001). Besides, OSNs housed in basiconic sensilla of male *R. prolixus* were found to be sensitive to several sex-pheromone components produced by metasternal glands, such as (S)-2-pentanol and 3-pentanol (Bohman et al. 2018).

Trichoid sensilla are hairs with a nonarticulated base and presenting a length of approximately 35 μm. Also named thick-walled wall-pore trichoid sensilla by some authors, they house 1 to 2 OSNs in *T. infestans* and up to 5 in *R. prolixus* (Wigglesworth and Gillett 1934; Bernard 1974; Catalá and Schofield 1994; Guerenstein and Guerin 2001; May-Concha et al. 2016). No odor stimulus tested so far was able to produce responses in OSNs housed in these sensilla (reviewed in Guerenstein and Lazzari 2010).

Grooved-peg sensilla are short double-walled wall-pore sensilla about 8–18 μm long and presenting a nonarticulated base. These short hairs normally house up to 5 OSNs (Wigglesworth and Gillett 1934; Bernard 1974; Guerenstein and Guerin 2001). Odor-triggered responses by grooved-peg OSNs vary between sensilla. Consequently, three different functional sensillum types have been identified in *T. infestans* (Diehl et al. 2003). All the three types, named GP1, GP2, and GP3, present an OSN that is excited by aliphatic amines such as methyl-, dimethyl-, trimethyl-, ethyl-, diethyl-amine, 2-butyl- and, isobutylamine. Besides GP1 and GP2 also contain one OSN activated by ammonia, but only GP2 houses an OSN that is excited by short-chain carboxylic acids like isobutyric, butyric, isovaleric, and 2-methylbutyric acids (Taneja and Guerin 1997; Guerenstein and Guerin 2001; Diehl et al. 2003; Bernard 1974).

3.2 Sensory Features of Olfaction

A total of 111 OR and 33 IR genes were reported in the genome of *R. prolixus* (Mesquita et al. 2015). Furthermore, expression of most ORs and IRs has been confirmed in the antennae of larvae and adults of this species (Latorre-Estivalis et al.

2016). Olfactory receptor deorphanization is highly desirable to establish which receptors mediate the detection of host odors and, aggregation, sex, and alarm pheromones, as they represent the molecular basis for such critical behavioral processes in triatomines. Even though triatomine bugs possess an exquisite ability to detect and orient to CO_2 (Barrozo and Lazzari 2004a, 2006; Barrozo et al. 2004; Bodin et al. 2008; Taneja and Guerin 1995), no orthologs of the well-characterized gustatory receptors acting as CO_2 receptors in *Drosophila melanogaster* have been found in the genome of *R. prolixus* (Robertson and Kent 2009). This suggests that other sensory proteins still to be characterized mediate CO_2 detection in triatomines. Two genes, *CIOR1* and *CIOR2,* mediate the detection of aliphatic aldehydes in the beg bug *Cimex lectularius* (Liu and Liu 2015). Interestingly, these genes are phylogenetically related to *R. prolixus*, *RproOR104* and *RproOR105*, respectively, suggesting that aldehydes with known behavioral or electrophysiological activity in triatomines may be the cognate ligands of these olfactory receptors, a hypothesis that deserves future evaluation. While most studies have examined responses at the peripheral level, not much is known about how odors are processed in the downstream olfactory centers of triatomines. Still, as in other insects, the axons of OSNs project to the primary processing center of olfactory information in the brain, the paired antennal lobes (ALs) (Barrozo et al. 2009). In *R. prolixus*, each AL is composed of 22 spheroid structures called glomeruli, without obvious sexual dimorphism (Barrozo et al. 2009).

4 The Taste Sense

The taste sense or contact chemoreception of triatomines is critical for food recognition and ingestion, but also likely in intraspecific communication. Food acceptance or rejection occurs after gustatory evaluation. Thus, this sensory modality allows organisms to detect the presence of appetitive compounds signaling nutritious food and promoting feeding, but also potentially noxious compounds finally eliciting aversion (Chapman 2003; Barrozo 2019). Besides, the taste sense plays a crucial role in mediating intraspecific communication. Mate recognition and assembling are often mediated, at least in part, by contacting nonvolatile compounds present in the cuticle of insects or in the surrounding substrate.

Taste sensilla bear a single apical pore through which chemical stimuli penetrate. Gustatory sensory neurons (GSNs) housed inside them can detect solid or liquid stimuli. Taste sensilla were described on the antenna, pharynx, and legs of triatomine bugs (Pontes et al. 2014; Barrozo et al. 2017). Up to date, there is no evidence of the presence of taste sensilla in the rostrum, maxillae, and mandibles of triatomines, as only mechanoreceptive structures have been reported in their mouthparts (Bernard 1974; Pinet 1968). There are three known types of taste sensilla in triatomines: antennal chaetic sensilla (previously named uniporous trichoids), legs chaetic sensilla, and epipharyngeal sensilla.

Antennal chaetic sensilla are long uniporous sensilla about 70–80 μm long presenting an articulated base can be found at the distal flagellomere (Pontes et al. 2014; Bernard 1974; Gracco and Catalá 2000; Insausti et al. 1999).

Leg chaetic sensilla are 20 μm long uniporous sensilla located at the tarsi and tibia of *R. prolixus,* which extend from a flexible socket (Barrozo et al. 2017).

Epipharyngeal sensilla are uniporous sensilla located in internal walls of the anterior region of the pharynx or epipharynx of triatomines. *R. prolixus* shows 8 short pegs about 2 μm long located inside a 2 μm diameter pit (Bernard 1974; Kraus 1957; Pontes et al. 2014). *T. infestans,* presents circa 11 semicircular domes with 1.5 μm diameter and 1.2 μm height. The latter have a single narrow opening that extends along with the dome (Bernard 1974; Kraus 1957; Pontes et al. 2014).

4.1 Sensory Aspects of Taste

Behavioral and electrophysiological studies revealed that antennal taste sensilla (and probably also chaetic taste sensilla present on legs) mediate host recognition and intraspecific communication. Epipharyngeal sensilla inform the brain about the quality of ingested food (Bernard 1974; Lorenzo Figueiras and Lazzari 1998b; Pontes et al. 2014; Barrozo 2019). However, further studies are needed to determine the number of GSNs inside antennal, leg, and epipharyngeal taste sensilla and to investigate the receptors mediating the detection of known tastants. In general, GSNs are tuned to a particular gustatory quality by means of membrane gustatory receptor proteins (GRs), ionotropic receptors (IRs), transient receptor proteins (TRPs), and pickpocket receptors (ppks) (Freeman and Dahanukar 2015). The activation of a single gustatory neuron is sufficient to elicit appropriate feeding decisions (Marella et al. 2006; Mueller et al. 2005). About 28 GRs, 33 IRs, 15 TRPs, and 10 ppk genes were identified in the genome of *R. prolixus* (Mesquita et al. 2015); many of their products potentially acting as taste receptors. Yet, the study of their functional role in the recognition of food sources or contact pheromones needs attention.

Once a host is found, triatomines taste the skin to assess its quality in order to decide whether or not to bite (Barrozo 2019). In the case of triggering a bite, the insect takes a blood sample for a second assessment in which certain phagoestimulants need to be detected to trigger the decision to eat to repletion (Barrozo 2019; Pontes et al. 2014; Friend 1965). Nevertheless, feeding in triatomines can be prevented by the detection of certain compounds that taste bitter to humans (Pontes et al. 2014). When *R. prolixus* evaluates the skin, taste sensilla present in the antennae and legs are probably responsible for the detection of caffeine and quinine (Pontes et al. 2014). Even though bitter compounds elicit similar aversive responses in *R. prolixus*, bugs can discriminate among them. This is likely performed by different taste receptors or internal signaling systems (Asparch et al. 2016). Similarly, even if high-NaCl doses (>0.5 M) provoke avoidance responses in these bugs

(Minoli et al. 2018), they are able to differentiate aversive doses of NaCl and caffeine, but not between NaCl and KCl (Masagué et al. 2020). Subsequently, epipharyngeal sensilla detect appetitive compounds such as adenosine triphosphate (ATP) and low-NaCl concentration (0.1–0.15 M), but also aversive compounds such as caffeine, quinine, berberine, salicin, and high-NaCl concentration (>0.2 M) (Pontes et al. 2014, 2017; Cano et al. 2017). ATP is the main phagostimulant for *R. prolixus* and *T. infestans* (Friend 1965; Guerenstein and Núñez 1994), although other nucleoside phosphates can also elicit gorging in *R. prolixus* with a considerably lower potency (Friend 1965; Friend and Smith 1971). However, the ATP receptor of triatomines and other blood feeding arthropods is still unknown. Interestingly, NaCl also acts as a phagostimulant, but only at adequate concentration, that is, 0.1–0.15 M (Pontes et al. 2017). Higher doses of NaCl (i.e., >0.2 M) elicit aversive feeding responses (Pontes et al. 2017), likely through the activation of a signaling cascade involving nitric oxide, soluble guanylate cyclase, and cGMP (Cano et al. 2017).

Besides, some triatomines exhibit a characteristic arrestment elicited by complex mixtures of long-chain hydrocarbons, n-alkanes, branched alkanes, and fatty acids (Juarez et al. 2001; Juarez and Fernandez 2007). These compounds are found on the cuticle of these insects and can be left on the substrate as footprints. Cuticular hydrocarbons also play a role in promoting sexual recognition in triatomines (Cocchiararo-Bastias et al. 2011). Females produce contact pheromones that mediate mate recognition in *T. infestans*, and likely, other triatomine species (Cocchiararo-Bastias et al. 2011). The identity and location of the taste sensilla involved in the detection of contact sexual signals still need to be addressed.

5 The Thermal Sense

The thermal sense of triatomines is considered extremely sensitive in the detection of the infrared radiation emitted by warm-blooded vertebrates (Lazzari 2009). This capability allows bugs to recognize a potential host and to estimate its size and distance-based solely on thermal information that is not affected by factors such as wind, which can disrupt conduction gradients and convective currents (Flores and Lazzari 1996; Lazzari and Nuñez 1989a, b; Schmitz et al. 2000; Lazzari 2009). Host-finding is not the only context in which triatomines use thermal information. In fact, these bugs use thermal cues for choosing resting places (Lorenzo and Lazzari 1999), synchronizing their circadian system (Lazzari 1992), and locating blood-vessels hidden under the skin of vertebrate hosts (Ferreira et al. 2007).

Thermal sensilla have no pores and seem less abundant than other antennal sensory structures. There are three types of structures tuned to sense heat in triatomine antennae: the ***coeloconic sensilla***, the ***tapered hairs***, and the ***cave organ*** (Barth 1952).

Coeloconic sensilla are short rounded pegs housed inside a 2 μm deep pit presenting 6 μm in diameter. In *T. infestans,* they distribute on the antennae, the legs, and different body parts, except on the proboscis (Bernard 1974; Ferreira et al. 2007), while in *R. prolixus* are only observed along the proximal flagellomere

(McIver and Siemicki 1985). There are about five to eight coeloconic sensilla *per* antenna (McIver and Siemicki 1985; Zopf et al. 2014a), each containing three putative thermoreceptive cells with unbranched dendrites (McIver and Siemicki 1985).

Tapered hairs are 14 μm long hairs having a diameter of 1.7 μm that extend close and parallel to the antennal surface between the pedicellum and the proximal flagellomere of *R. prolixus* (Zopf et al. 2014a). There are six to eight tapered hairs in each antenna of this species.

Cave organ is an internal cuticular invagination, which terminates inside an ellipsoidal cavity. This structure is located in the pedicellum of triatomines (Barth 1952; Lazzari and Wicklein 1994), presenting a highly folded cuticle at its opening and extending internally through a 90 μm long channel ending in a cavity covered by numerous hairs of variable length (Barth 1952; Catalá 1994; Lazzari and Wicklein 1994).

5.1 Sensory Aspects of Thermoreception

Thermoreceptive cells are commonly present together with hygroreceptors, forming a triad of sensory cells that either respond to temperature decreases, dryness, or wetness (Altner and Loftus 1985; Steinbrecht 1998). This is the case for the coeloconic sensilla of *T. infestans* that house one neuron that increases activity with decreasing temperature, named cold cell, and two neurons that increase or decrease their firing activity with humidity changes, named dry and moist cells (Bernard 1974). Heat and humidity emitted by the skin or with the breath of warm-blooded animals are attractive to triatomines. In fact, both *T. infestans* and *R. prolixus* have the ability to detect water vapor (Wigglesworth and Gillett 1934; Barrozo et al. 2003). Moreover, because humid air increases thermal conductivity, moist heat sources can be detected from a greater distance than dry ones (Barrozo et al. 2003). Yet, thermal responses can also be enhanced by bimodal convergence of peripheral thermosensitive and hygrosensitive inputs in the central nervous system (Lazzari 2009).

Differently, the thermoreceptor sensilla in *R. prolixus* house one warm and one cold cell as reported in different insects (Altner and Loftus 1985; Gingl and Tichy 2001; Zopf et al. 2014a, b). Thus, a warm and a cold cell can be found inside coeloconic sensilla and tapered hairs of *R. prolixus* (Zopf et al. 2014a). In addition, a third neuron within these sensilla responds to increases in humidity (Zopf et al. 2014a). Both cold and warm receptor cells show stable, nonadapting, spiking activity in response to stimuli with constant intensity (Zopf et al. 2014a). This was also observed in the cold, dry, and moist cells of *T. infestans* (Bernard 1974). In *R. prolixus*, the firing rate of the warm cells increased in response to air-temperature increases, while the opposite was observed in cold cells (Zopf et al. 2014a). Thermoreceptor cells within both coeloconic and tapered-hairs also respond to IR pulses in still air (Zopf et al. 2014a). However, tapered-hair cells can also be stimulated by moving warm air, convective air, and IR pulses, and exhibit stronger responses than thermoreceptors housed in coeloconic sensilla (Zopf et al. 2014a). It

seems that triatomines use mechanical flow information to discriminate between convective and radiant heat (Zopf et al. 2014a). The coexistence of mechanoreceptive cells and thermoreceptors inside the same sensillum further supports this idea (Altner and Loftus 1985).

Regarding the sensory proteins mediating thermosensation, evidence indicates that the *Rprolav* gene, which is an ortholog of the mammalian cation channel TRPV is expressed in the antennae and other body parts of *R. prolixus*, and mediates heat detection (Zermoglio et al. 2015). Other potential heat receptors, like the *R. prolixus* ortholog of TRPA1, might also be involved in the detection of warmth in bugs, as shown for mosquitoes and fruit flies (Corfas and Vosshall 2015; Hamada et al. 2008; McMeniman et al. 2014). Besides, the genes named *waterwitch* and *nanchung* have been shown to mediate the detection of moist and dry air in fruit flies (Liu et al. 2007) and their orthologs were also identified in *R. prolixus* (Mesquita et al. 2015).

6 Mechanoreception

Mechanosensation mediates different aspects of the biology of triatomines. These bugs are extremely sensitive to air movement, and it probably plays a role in orientation toward odor-laden currents. Triatomines produce vibratory signals, through stridulation, to communicate during mating attempts and under mechanical disturbance. These signals propagate as mechanical waves through the insect body and substrate (Lazzari et al. 2006). Mechanoreception also mediates feeding and participates in texture evaluation during biting attempts (Ferreira et al. 2011). Finally, the tendency of these bugs to hide inside narrow shelters is partly mediated by their intense thigmotaxis (Mosquera and Lorenzo 2020).

Mechanoreceptive sensilla are the most abundant sensory structures found along the whole body of the insect. Yet, even if they are numerous, physiological evidence about their roles in known behavioral contexts is actually insufficient. Five morphological types of mechanosensilla exist in the antennae of triatomines: trichobothria, bristle sensilla (types I and II), campaniform sensilla, and tapered hairs.

Trichobothria are slender hairs about 60–210 μm in length that stand in large cup-like depressions, present on the lateral side of the pedicellum and the basal region of the proximal flagellomere (Catalá and Schofield 1994; Lent and Wygodzinsky 1979; McIver and Siemicki 1985). The number varies between 5 and 9 *per* antenna in adults, but there is only 1 close to the end of the pedicellum in nymphal stages (Catalá and Schofield 1994). Each sensillum is innervated by a single bipolar neuron and two sheath cells (McIver and Siemicki 1985).

Bristles (types I and II): The length of type I bristles varies from 38 to 150 μm, these hairs are present on the scape, pedicellum, and basal third of the proximal flagellomere. The length of type II bristles varies from 60 to 120 μm and they are only found on the distal part of the antenna (i.e., distal two thirds of the proximal flagellomere and all along the distal flagellomere). The shaft of type I bristles is curved toward the antenna and grooved and serrated distally, whereas type II hairs

are straight forming an angle of 50°–70° to the flagellar shaft (McIver and Siemicki 1985; Catalá and Schofield 1994).

Campanifom sensilla are not numerous oval domes surrounded by a ring of raised cuticle, present on the pedicellum, proximal flagellomere, and the scape (McIver and Siemicki 1985; Catalá and Schofield 1994).

Tapered hairs are conical shafts of 12–30 μm in length, found at the distal edge of scape, pedicellum, and proximal flagellomere (McIver and Siemicki 1985; Catalá and Schofield 1994). About six to eight tapered hairs (12 μm) are found at the proximal flagellomere and point distally. Longer tapered hairs (30 μm) project laterally from the base of the pedicellum. Presumably, they are innervated by one mechanosensitive bipolar neuron (McIver and Siemicki 1985).

Johnston's organ is an internal structure within the antennae, located at the distal end of the pedicellum, composed by scolopidial cells (Wigglesworth and Gillett 1934).

Other types of mechanoreceptors are the scolopidial organs of the tibia (Autrum and Schneider 1948) and the mandibular mechanoreceptors (Bernard 1974).

6.1 Sensory Aspects of Mechanoreception

In triatomines, anemotactic responses triggered by air movement are probably mediated by trichobothria (Lent and Wygodzinsky 1979), and the Johnston's organ (Wigglesworth and Gillett 1934) of the antennae, even though to date there is no experimental evidence supporting such claim. Antennal bristles have a tactile role. Type I bristles are likely friction detectors due to their curved serrated endings, while type II bristles probably sense the environment while the antennae touch different surfaces (McIver and Siemicki 1985). Campaniform sensilla probably function in proprioception, monitoring cuticular stress, and antennal positioning. It is possible that tapered hairs provide information about the relative position of the antennal segments (Insausti et al. 1999), also acting as proprioceptors. Yet, tapered hairs have also been found to have thermoreceptive function (see above).

Triatomines stridulate producing a sound by rubbing the tip of the proboscis with a longitudinal groove on the prosternum. Because triatomines have no discrete tympanal organs or subgenual organs, it was proposed that this sound produced during stridulation must be perceived with the Johnston's organs of the antennae (Schofield 1977). However, other authors have suggested that stridulatory vibration may represent substrate-borne signals transmitted either by the substrate or the body itself (Manrique and Lazzari 1994; Manrique and Schilman 2000; Lazzari et al. 2006) and therefore, they would probably be perceived through scolopidial organs located in the tibia (Autrum and Schneider 1948). Nevertheless, this remains to be investigated experimentally.

The texture properties of the substrate, probably evaluated by mandibular mechanosensory receptors located in the proboscis, are relevant to induce bug probing after biting warm objects (Ferreira et al. 2011). In this task, the Johnston's organ of

the antenna, which is connected to the base of the mandibles, could be involved by controlling the penetration of the stylet into the host skin (Barth 1953). In addition, mandibular mechanoreceptors at the distal region of the mandibles are known to respond to the deflection of the stylets. Consequently, they have been implicated in the control of stylet penetration during blood vessel piercing (Pinet 1963; Bernard 1974).

Even if this sensory system seems to be relevant for the sensory ecology of triatomines, it remains by far one of the least studied and most of the sensory aspects reviewed here are presumptive.

7 Concluding Remarks

Knowledge about how triatomines perceive the world is relevant for understanding their general sensory biology, likely common to many insect species. But it is probably more important to design new control strategies targeting their sensory systems. The new era of functional genetics of insect behavior should open opportunities for the development of target-specific interference control tools such as chemical agents inhibiting the function of key receptors. Modulation of sensory function through the control of receptor gene expression, or that of other relevant sensory genes, may be an alternative. The development of agents that manipulate sensory abilities seems to represent a potential task that deserves deeper study in the future.

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The Behaviour of Kissing Bugs

Claudio R. Lazzari

Abstract Many arthropod species have adopted the blood of vertebrates as their main food. Blood is rich in nutrients and, except for the presence of parasites, otherwise sterile. This food, however, is not freely available nor is its obtention devoid of risk; it circulates inside vessels hidden underneath the skin of mobile hosts, which are able to defend themselves and even predate the insects attempting to feed on them. Thus, the haematophagous lifestyle is associated with major morphological, physiological and behavioural adaptations that have accumulated throughout the evolutionary history of the various lineages of blood-sucking arthropods, including triatomines. These adaptations have, on the other hand, significant consequences for the evolution of parasites, as well as for the epidemiology of vector-transmitted diseases. This review article analyses various aspects of the behaviour of triatomine bugs to illustrate how behavioural traits represent particular adaptations to their close association with hosts, which may easily turn into predators. The aim is to offer to the reader an up-to-date integrative view of the behaviour of Chagas disease vectors from a personal perspective, with the hope of encouraging young and experienced colleagues to explore this fascinating aspect of triatomine biology.

Keywords Host search · Communication · Chronobiology · Pheromones · Orientation · Sexual behaviour · Aggregation

1 Introduction

Kissing bugs, in particular *Rhodnius prolixus,* exhibit the double interest of being considered as a classical model in insect physiology and also a vector of a major health problem in the New World, that is, Chagas disease. Understanding the behaviour of kissing bugs, as well as its underlying physiological mechanisms, is crucial

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for better understanding their role as vectors of *Trypanosoma cruzi* and may be adapting strategies for better controlling the transmission of Chagas disease.

Not far in the past, it was sometimes laborious persuading certain scientific and non-scientific fora about the necessity of a deep biological knowledge for designing and adapting efficient control campaigns. The reason was a fair point: the urgency for stopping disease transmission masked the necessity of scrutinising fundamental aspects. Control campaigns have been relatively successful and important progress has been achieved in eradicating bugs from vast regions. Yet, some problems, such as the development of resistance to control actions and resilient house reinfestation, still require digging deeper in biological aspects; not in the search for a 'silver bullet', but for making control as effective as possible.

In recent years, much effort has been devoted to the study of triatomine behaviour. This interest is not only inspired by the need to control bugs but also because these insects constitute an excellent model for the study of fundamental aspects of haematophagy and general questions on insect physiology, which have been pondered since the time of V.B. Wigglesworth. The present document presents an analytical review of our knowledge about triatomine behaviour and suggests possible future research topics regarding particular aspects for which a better understanding would be beneficial. Besides, the author is convinced that triatomines, and in particular *Rhodnius prolixus*, still constitute excellent model systems for unravelling fundamental biological mechanisms. Recently, the genome of *Rhodnius prolixus* has been sequenced (Mesquita et al. 2015), representing a major breakthrough in our comprehension of the genetic bases of the adaptations of insects to haematophagy and the interaction of blood-sucking insects with parasites and hosts. This achievement opened multiple research lines on lesser understood biological aspects and gave place to novel hypotheses, many of which wait for a functional validation. The timing for this review seems appropriate to open novel research possibilities in functional genetics of insect behaviour.

Most of our current knowledge on the behaviour of kissing bugs comes from the exhaustive study of a group of species considered as major vectors of Chagas disease, in particular *Rhodnius prolixus* and *Triatoma infestans*, but also *Panstrongylus megistus*, *Triatoma brasiliensis*, *Triatoma dimidiata* and few others. Yet, given the diversity of species, habitats and hosts associations present in the group, we can expect differences in the behavioural adaptations across triatomine species, in particular those inhabiting isolated ecotopes or challenging to rear in the laboratory. As a consequence, the information presented here should not be taken as valid for every species of the subfamily, but as representative of well-established strategies and mechanisms, serving as a rational basis for the analysis of behavioural traits and adaptations of other species to their specific way of life. An extensive revision of the behaviour of triatomines has been published some years ago (Lazzari et al. 2013); this chapter presents a more synthetic and updated view.

2 Host Search and Feeding Behaviour

Triatomines are documented as nocturnal insects that during daylight hours remain hidden and virtually immobile (i.e. akinesis) in dark places. During the first hours after dusk, bugs awake and begin exploring the surroundings, looking for distinctive cues revealing the presence of warm-blooded vertebrates. Host detection is achieved via air currents carrying distinctive odours, water vapour and heat. For this, triatomines exhibit not only a high level of sensitivity, but also the capacity of integrating multimodal information. Conversely, inhibitory mechanisms keep the insects away from hosts when obtaining a blood meal that is not absolutely necessary (Guerenstein and Lazzari 2009).

2.1 Orienting Cues

When insects are motivated to search for a resource, as a hungry bug leaving its refuge to search for food, the sensory cues associated with that resource are not necessarily apparent. Most insects do not sit and stay until something appears, nor move randomly looking for signs. Instead, they orient themselves relative to air currents potentially transporting odours of interest. The way insects use air currents varies according to the stability of their direction (Sabelis and Schippers 1984; Bau and Cardé 2015). Even though inside a nest or house one cannot expect intense winds, air always moves (e.g. convective currents). Upon detection of host-associated cues, appetitive search and long-range orientation are triggered (Barrozo et al. 2017). It should be noted that, when detected at a distance, olfactory cues do not provide directional information by themselves, but they can trigger instead positive anemotaxis, that is, upwind displacement (Guerenstein and Guerin 2001; Guerenstein and Lazzari 2009; Núñez 1982; Taneja and Guerin 1995). When detected at a close range, chemical cues can stimulate the insects to follow the concentration gradient and attract them. As in virtually every blood-sucking insect, carbon dioxide modifies the behaviour of triatomines (Barrozo and Lazzari 2004a; Guerenstein and Hildebrand 2008). Triatomine responses to carbon dioxide are modulated along the day by an endogenous circadian rhythm, making insects responsive when feeding motivation is also higher during the first hours of the scotophase (Barrozo et al. 2004a; Bodin et al. 2008; Lorenzo and Lazzari 1998). The responsiveness of triatomines to carbon dioxide is also dependent upon their physiological condition. Long starvation periods do not seem to strengthen the response (Barrozo and Lazzari 2004a), but recent feeding switches the effect of carbon dioxide from attraction to repellence (Bodin et al. 2009b). Moulting and reproduction (Bodin et al. 2009a, b) also affect the responses of bugs to carbon dioxide. In contrast to other haematophagous insects, carbon dioxide is not an essential component for the attraction of triatomines, although air currents loaded with relatively low quantities of carbon dioxide are able to evoke attraction by themselves (Barrozo and

Lazzari 2004a). Some host odorants, such as nonanal, increase bug activity (Guerenstein and Guerin 2001) and others that are found in human sweat (Cork and Park 1996) such as isobutyric acid and 1-octen-3-ol induce their attraction (Barrozo and Lazzari 2004b; Guerenstein and Guerin 2001). Finally, ammonia, which is present in the sweat and urine of vertebrates, is able to evoke both activation and attraction in *Triatoma infestans* (Taneja and Guerin 1997), antennal responses to this compound being higher with increasing starvation (Reisenman 2014).

In nature, kissing bugs are confronted with mixtures of odours rather than with isolated volatiles. The components of such mixtures often interact in a synergistic form. In the case of triatomines, the response threshold for pure carbon dioxide is beyond 300 ppm above atmospheric concentration (Barrozo and Lazzari 2004a). Interestingly, pure L-lactic acid and short-chain fatty acids that are present in human sweat and on the skin (Bernier et al. 2000; Cork and Park 1996) are completely ineffective for triggering anemotaxis over a wide range of concentrations (Barrozo and Lazzari 2004a, b). Nevertheless, when sub-threshold amounts of carbon dioxide are combined with L-lactic acid and fatty acids in appropriate proportions, the attractiveness of the mixture becomes similar to that of a living host (Barrozo and Lazzari 2004b). Recently obtained experimental evidence supports the idea that microorganisms associated with human skin produce volatiles that are attractive to *Rhodnius prolixus* (Ortiz and Molina 2010; Tabares et al. 2018). These findings add a novel dimension to the analysis of how Chagas disease vectors recognize and localize their hosts.

Even though both vision and vibro-reception are well-developed triatomine senses, so far there is no evidence suggesting a role in host orientation. Mechanoreception is involved in long-range orientation, but not in relation with hosts, but with the detection of air currents guiding upwind displacement. The most important host-associated physical cue perceived by triatomine bugs is heat. Even if the heat emitted by host bodies is widely used as an orienting cue for many blood-sucking insects, its role remains relatively unknown for the vast majority (Lazzari 2009, 2019). In contrast, the thermal sense of triatomines has been the object of detailed studies. The sensitivity of triatomines to heat is extremely high, such that the insect can detect differences of thermal energy in the order of a few μWatt/cm^2 (Lazzari and Núñez 1989b; Lorenzo et al. 1999a, b; Lazzari 2009). It has been calculated that *Triatoma infestans* is capable of detecting the heat emitted by a human face from a distance of approximately 2 m and by a mammal the size of a dog from several meters (Lorenzo et al. 1999a, b).

Triatomines remain the only group of blood-sucking insects whose ability to perceive host-emitted infrared radiation has been demonstrated (Lazzari and Núñez 1989b; Schmitz et al. 2000). This ability has important implications for successful host finding because infrared radiation propagation is not disrupted by air currents (which do disrupt conductive and convective heat transfer) or by the relative position of the insect with respect to the thermal source (which does influence convective heat transfer) (Lazzari 2009, 2019).

A remarkable characteristic of *Triatoma infestans* is the ability to detect the temperature and distance of heat sources, independently of their sizes, and based on

thermal information alone. This seems to be possible due to their capacity to perceive radiant heat (Lazzari 2009; Lazzari and Núñez 1989b). A hypothetical model on how these bugs distinguish the temperature, size, and distance of objects based on thermo-receptive inputs and active antennal movements has been proposed by Lazzari (2009). It provides an explanation for the ability of triatomines to discriminate distant (or small) burning objects from closer (or large) tepid ones, even if the amount of thermal energy reaching their antennae is of the same magnitude.

The extreme thermal sensitivity of triatomines, together with their ability to discriminate the temperature of distant objects, raises the question about whether the absolute temperature of a heat source and the difference between the temperature of objects and their background are in fact used by these insects to recognize their hosts. This question is related to another, more practical one: are triatomines able to perceive objects at the temperature of a host when the ambient temperature is higher? Experiments were performed for testing the response of bugs to an object presented at different temperatures, in a chamber providing a background temperature that could also be modified (Fresquet and Lazzari 2011). Bugs responded by trying to bite objects showing temperatures between 30 °C and 40 °C, but only if the surrounding environment was colder than the objects themselves. Therefore, their ability to measure the temperature of objects is only effective when a positive difference exists between object and air temperature.

It should be emphasized that, in a natural context, triatomines are not exposed to single cues such as specific odours or heat, but to multiple cue combinations detected by different sensory modalities (mechanical, chemical, thermal and hydric). In the context of food search, multimodal convergence may increase the ability of bugs to find a host. For instance, it has been shown that water vapour, which constitutes a close-range orientation cue by itself, also increases bug responsiveness to heat (Barrozo et al. 2003). This can be due to the convergence of different sensory inputs into the insect brain or to a physical phenomenon (i.e. moist air transports more heat than dry air) increasing sensory stimulation.

2.2 Orientation Mechanisms

Sensory information can be used in different ways for locating a resource. In some cases, the source of stimuli is the resource itself, as the colour of a flower, but in others, external cues become relevant. For instance, the sun, the moon and the polarization pattern of the sky guide the displacements of ants, bees, beetles and other insects. In a similar way, the wind plays a major role in the orientation of insects that follow odour tracks. This is necessary because not all stimuli provide spatial information in the same way, nor the sensory system is able to extract precise spatial information from them. It is possible to locate a light spot in the night without difficulty, even with just one open eye, because light produces a discrete spatial pattern, propagates radially, it is not altered by air currents and our eyes are capable of

producing a detailed image, even monocularly. This is not the case, however, for all potential stimuli, nor for all sensory systems (Lazzari 2009).

If we consider two main cues emitted by hosts, that is, odours (kairomones) and heat, each modality presents its own particularities and requires specific mechanisms in order for them to serve as orienting cues.

Odours disperse in the air and are transported by the wind and, as a consequence, only provide spatial information in the close proximity of the source. Insects, in general, and kissing bugs, in particular, use different mechanisms for olfactory orientation, according to the situation. Those chemical stimuli relevant for locating and recognizing elements in close proximity or physical contact usually act in high concentration and form stable concentration gradients, which can be followed by the insect. For instance, this is the way chemical tastants on the host skin are recognized before deciding to bite or not (Barrozo 2019). On the other hand, the detection of volatiles emitted by a distant host does not provide enough spatial information. In that case, upon detection of host-associated chemicals, bugs turn upwind, following the direction of the air current transporting the odour.

Heat disperses radially as light, but there is no 'thermal retina' for its detection, but specialized receptors located in the antennae (Zopf et al. 2014a, b). Then, from the point of view of a kissing bug, the spatial information delivered by a warm object is not as precise as that of a light source, but nor as imprecise as that of a source of odours. As indicated above, triatomines use thermal cues for locating a host and, once in contact with its skin, to locate the most adequate spot to be bitten (see below). Interestingly, each task involves a different use of sensory information or orientation mechanism (Wigglesworth and Gillett 1934, Flores and Lazzari 1996). The approach to a warm object can be performed using just one antenna and the bugs can determine the direction and follow thermal gradients for approaching, using a mechanism called 'telotaxis' (i.e. enough information is provided by only one bilateral organ). Yet, when they extend their proboscis, bilateral inputs from both antennae need to be integrated in order to reach the target precisely, that is, 'tropotaxis' (i.e. the contribution of both bilateral organs is necessary, Flores and Lazzari 1996; Lazzari 2009,).

The consideration of these possible alternatives (i.e. orientation mechanisms) in the use of sensory information is crucial in the design of experiments. For instance, the use of olfactometers using air currents for transporting odours is a very usual way for testing the response of insects to odours, kissing bugs included. Yet, this type of device is conceived to evoke odour-triggered anemotaxis, but this is not the only way a chemical compound may attract an individual, and a negative result does not necessarily mean that the insect does not respond or does not detect the odour. Triatomines do not respond to their arresting pheromone left on walked substrates (Lorenzo Figueiras and Lazzari 1998b).

2.3 Biting and Feeding

In addition to its role as an orienting signal during host search, heat is also used to locate blood vessels hidden under the host skin (Ferreira et al. 2007). By analysing the bugs feeding behaviour on live hosts, it has been shown that the insects do not bite randomly; instead, they extend their proboscises mostly towards vessels. When the host skin was replaced by a vessel-shaped heat source placed on a heated metal plate, both with independently controlled temperature, a similar precisely directed choice was observed for the warmest linear area. This suggests that heat discontinuities over the skin surface area are used to guide the bite. Biting the warmest patch of skin surface requires a bilateral integration of the thermal inputs provided by both antennae. If this integration is experimentally altered by a unilateral or bilateral antennectomy, bugs either miss the target (unilateral) or do not exhibit proboscis extension responses at all (bilateral antennectomy). This suggests that, if present, rostral thermoreceptors do not provide information for triggering or guiding biting (Ferreira et al. 2007). Interestingly, heat is a key factor for host finding and biting, but once a blood is contacted, heat becomes no longer relevant (Lazzari and Núñez 1989a, b).

The decision to bite the host skin and ingest its blood is a complex process for triatomines. Classical work revealed that food recognition in triatomines is based on the analysis of specific food properties, such as osmolarity (Guerenstein and Núñez 1994) and the presence of phagostimulant compounds (Friend and Smith 1977). For a long time, this remained the only information available. Yet, recent work has shed supplementary light on the process, revealing an elaborated feeding decision-making system, depending on specific sensory pathways and cognitive processes (Barrozo 2019).

Not all triatomines are obligate haematophagous insects. Entomophagy has been frequently reported in different species of kissing bugs. This habit, which can also be expressed towards conspecifics, involves either haemolymphagy or cleptohaematophagy (i.e. stealing part of the vertebrate blood ingested by a conspecific). For instance, these phenomena have been reported to occur in *Triatoma infestans* and in *Rhodnius prolixus* (Schaub et al. 1989; Alves et al. 2011; Lazzari et al. 2018), eventually resulting in direct *Trypanosoma cruzi* transmission amongst Chagas disease vectors (Schaub 1988). Interspecific entomophagy is also frequent (Garrouste 2009; Pontes et al. 2011; Duran et al. 2016). Even though haemolymphagy and cleptohaematophagy can be considered two forms of cannibalism; there are different processes. Cleptohaematophagy is usually intraspecific and the gathered food is similar to that obtained from a vertebrate host, that is, blood. Haemolymphagy can occur against conspecifics or heterospecifics and the food is not vertebrate blood. It can be concluded then that the triatomine saliva is not only adapted to gathering blood from vertebrates, but also insect haemolymph (Alves et al. 2011).

Intriguingly enough, recent reports describe *Rhodnius prolixus* consuming a sugar solution (Diaz-Albiter et al. 2016) and water from a drop (Páez-Rondón et al.

2018) in the laboratory. These observations deserve to be further investigated, in order to evaluate their biological implications for triatomines.

3 Sexual Behaviour

Mating is a major biological necessity and insects have developed complex mechanisms for locating, attracting and choosing potential partners. Triatomines are not an exception and, even though no elaborated courtships can be observed as in other insects, mating relies on specific interactions between male and female individuals. One of them is polyandry, which has been observed in females of different bug species (Baldwin et al. 1971; Manrique and Lazzari 1994, 1995; Vitta and Lorenzo 2009; De Simone et al. 2018).

One aspect deserving major interest is the chemical attraction between sexes. The study of sexual pheromones has been particularly challenging in triatomines, with respect to their origin, biological significance and composition. Despite a considerable amount of experimental work and published observations on the chemical ecology of triatomines (Cruz-Lopez et al. 2001), it took time to understand how sexual pheromones work in this group. The first evidence of their occurrence in triatomines was obtained in *Rhodnius prolixus* (Baldwin et al. 1971) and much later in *Triatoma infestans* (Manrique and Lazzari 1995). In these species, mating couples release volatiles that attract and gather males, who eventually mate with a single female.

The origin of sexual volatiles has been a matter of controversy due to early reports describing the detection of isobutyric acid, produced by Brindleys glands, in the 'headspace' (i.e. air surrounding) of mating pairs (e.g. Fontan et al. 2002). It is worth highlighting that this compound is usually released when adult triatomine bugs are disturbed (Barrett et al. 1979). However, experiments occluding different exocrine glands of males and females alternatively revealed that female metasternal glands are the source of sexual signals, but not Brindleys glands (Crespo and Manrique 2007; Pontes et al. 2008; Vitta et al. 2009). Chemical analysis subsequently confirmed that isobutyric acid is not present in metasternal glands (Manrique et al. 2006) and does not make part of the sexual signal. Further evidence supports that female metasternal glands odours are emitted preferentially during the scotophase (Pontes et al. 2008), and that the pheromone is capable of inducing males to leave their shelters (Pontes 2010), take flight (Zacharias et al. 2010), aggregate (Pontes and Lorenzo 2012) and display odour-modulated anemotaxis in airstreams associated with female odours (Vitta et al. 2009; Pontes et al. 2014; May-Concha et al. 2013). The secretion of metasternal glands can be a complex mixture eventually including ketones, alcohols, dioxolanes and aldehydes (Manrique et al. 2006; Pontes et al. 2008; Vitta et al. 2009; May-Concha et al. 2013). Chemical and behavioural analyses allowed the recent development of a synthetic female-pheromone blend, capable of attracting males of *Rhodnius prolixus* in the laboratory (Bohman et al. 2018).

Additionally, female cuticular hydrocarbons seem to play a role in mate recognition (Cocchiararo-Bastias et al. 2011).

Non-receptive females reject male copulatory attempts by displaying various evasive behaviours including stridulation (Manrique and Lazzari 1994). Although differences have been reported for the mating behaviour of various triatomine species, stridulation by unreceptive females during male mating attempts seems to be a frequent feature (Lazzari et al. 2006).

4 Aggregation and Alarm

Triatomine bugs are gregarious insects sharing shelters. Depending on the species, this behaviour can be mediated by two chemical signals: a volatile aggregation pheromone emitted by bug faeces and a cuticular contact factor deposited on substrates. These signals act parallelly to the known tendency to maintain physical contact with the substrate and conspecifics (thigmotaxis) and by visual cues (i.e. darkness).

The faeces of triatomines are a source of aggregation pheromones that attract and gather bugs in their proximity (Lorenzo Figueiras et al. 1994; Schofield and Patterson 1977). It was originally suggested that the functionality of this signal would be to indicate a suitable food source (Schofield and Patterson 1977). Further studies revealed that faeces accumulate in the access path to refuges and play a major role as chemical landmarks for finding shelters (Lorenzo and Lazzari 1996). The pheromones emitted by bug excrement do not seem to have species-specific effects as they are capable of assembling bugs of other species (Lorenzo Figueiras and Lazzari 1998a; Pires et al. 2002a, b, c). They can be extracted using polar solvents, and chemical analysis has revealed multiple compounds whose biological role is not yet clear (Alzogaray et al. 2005; Cruz-Lopez et al. 2001; Mota et al. 2014).

As said above, bugs of some species deposit on substrates a non-volatile footprints signal promoting aggregation. This signal can be extracted with non-polar solvents and seems to be composed by a mixture of hydrocarbons of cuticular origin (Lorenzo Figueiras et al. 2009) and insects require physical contact for its detection (Lorenzo Figueiras and Lazzari 1998b). This pheromone, therefore, can be considered to be an arresting factor rather than an attractive one. It is worth noting that *R. prolixus* and *T. brasiliensis* do not seem to use footprints to promote arrestment (Lorenzo Figueiras and Lazzari 2002; Vitta et al. 2007), suggesting that mechanisms promoting bug aggregation differ across species.

Bugs exhibit a tendency to avoid illuminated environments, that is, negative phototaxis, which is mediated by the compound eyes and, in adult bugs, also by the ocelli (Lazzari et al. 1998; Reisenman et al. 1998). This photophobic behaviour also mediates shelter choice (Reisenman et al. 2000).

It is a frequent observation that mechanically disturbed adult triatomines release a pungent odour, which origin is the secretion of their Brindleys glands. As indicated above, the secretion is composed of isobutyric acid, together with a complex

mixture of other volatiles (Cruz-Lopez et al. 2001; Guerenstein and Guerin 2004; Manrique et al. 2006; Palottini and Manrique 2016) produced by these glands. Triatomines respond by walking away from conspecifics releasing the Brindleys glands secretion (Manrique et al. 2006); yet, some reports also indicate that bugs approach to sources releasing low quantities of isobutyric acid (Ward 1981; Guerenstein and Guerin 2001). This dual response has not facilitated the distinction of the biological significance of this compound in triatomine biology. Its release by physically disturbed bugs and its repulsive role on other individuals have allowed considering the secretion of Brindleys glands as an alarm pheromone. It should be noted that Brindleys and metasternal glands are only present in adults. Given that the latter are involved in sexual communication, its exclusively imaginal character can be easily understood. Nevertheless, the fact that nymphs could respond to, but not produce alarm pheromones, remains to be interpreted in adaptive terms. As in other insect species, the response of bugs to their own alarm pheromone is modulated by the individual experience (Minoli et al. 2013).

5 Learning and Memory

In the past, insects were considered to be 'reflex machines' whose behaviour were mostly ruled by stereotyped, innate responses to external stimuli (Giurfa 2004). At present, their ability to adapt their behavioural responses as a function of their previous experience is widely recognized. Despite possessing tiny brains and much less neurons than vertebrates, they have largely proven to be capable of complex forms of learning, including the acquisition of rules and concepts (Giurfa 2003; Giurfa et al. 2001). As a consequence, insects became major models in the study of learning and memory. Most studies in this area have focused only on a handful of species, and particularly, on vinegar flies and honeybees. Provided that none of them presents a haematophagous habit, the selective pressures that have modelled their cognitive abilities are much different to those acting on blood-sucking arthropods. Yet, the potential learning abilities of disease vector insects may have significant epidemiological consequences, because they can induce heterogeneous biting patterns within host populations (Kelly 2001; Kelly and Thompson 2000). Due to the strong selection pressures to which blood-sucking insects are exposed, it is reasonable to ask to what extent their cognitive abilities are developed and how could they modulate vector behaviour. For instance, learning to recognize the most vulnerable hosts through individual experience could influence host selection and, as a result, affect parasite transmission patterns (McCall and Kelly 2002; Vinauger et al. 2016).

Many field and semi-field studies have evinced behavioural responses that might indicate learning capabilities in disease vectors. Nevertheless, only a few controlled studies have succeeded in demonstrating conclusive experimental evidence (Alonso and Schuck-Paim 2006; Alonso et al. 2003).

Amongst Chagas disease vectors, early experiments failed to demonstrate learning capabilities (Abramson et al. 2005; Aldana et al. 2005, 2008). In most cases, the

absence of positive results can be associated with issues that often plague this kind of studies. Learning experiments need a rigorous control over experimental variables (insect motivational state, time of the day etc.), adequate neutral stimuli (i.e. unquestionably perceived by the insect, but ineffective in evoking the evaluated response) and finally, a deep knowledge of the experimental model. It should also be noted that experimental psychology imposes a strict theoretical framework and numerous control experiments in order to properly assess the learning capabilities of an animal. In contrast to the study of honeybees and *Drosophila*, this has not always been respected in studies dealing with disease vectors (Alonso and Schuck-Paim 2006). In many cases 'learning-compatible' results are considered as evidence of learning to occur, without adequately excluding other, more parsimonious, explanations.

Triatomines are adequate models for the experimental study of the cognitive capabilities of blood-sucking insects because: (1) they can be reared in the laboratory under controlled conditions and fed using artificial feeders; (2) they are haematophagous during their whole life, thus facilitating experimentation with juveniles and adults; (3) their size facilitates testing them in constrained conditions (i.e. fixation to a holding support or to a locomotion compensator); (4) they express a widely exploited response in insect learning studies, the *proboscis extension reflex* or PER. Last but not least, our knowledge about the behavioural biology and the sensory ecology of triatomines is one of the deepest amongst disease vectors. This knowledge allows investigating cognitive abilities in meaningful biological contexts, such as blood feeding, aggregation and escape.

One of the most generalized forms of learning is establishing novel associations between pairs of stimuli by classical or Pavlovian conditioning. In its most basic form of classical conditioning, a 'neutral' stimulus, known to be perceived by the animal, but unable to trigger a given response (e.g. orientation) by itself, is paired with another stimulus, the 'unconditioned stimulus', which innately evokes the desired response. After presenting them together several times, the originally neutral stimulus may acquire the capacity of evoking the same response, turning in what is called a 'conditioned stimulus'.

Classical conditioning protocols have been adapted for the appetitive and aversive training of *Rhodnius prolixus*. Vinauger et al. (2011a, b) successfully conditioned bugs to the same neutral odour, either making it act as an appetitive (i.e. inducing attraction) or aversive (i.e. inducing avoidance) conditioned stimulus. In that study bugs learnt to associate lactic acid, which is perceived but does not evoke any oriented behavioural response by itself in bugs, with either a blood meal or with mechanical disturbance. After being trained to reinforce a specific association, bugs were confronted with an air current loaded with lactic acid to test whether their innate behaviour changed. Not surprisingly, they manifested either significant attraction or significant repulsion to lactic acid-associated air currents, depending on their previous appetitive or aversive individual experience. This demonstrates not only the ability of bugs to learn and to remember information, but also their ability to use that knowledge in different contexts, for example, use in orientation tests in

an olfactometer, what they have learnt when confronted to an artificial feeder (Vinauger et al. 2011a, b).

The ability of triatomines to modify their responses to chemical stimuli as a function of their individual experience has been proven to affect their host choices (Vinauger et al. 2012).

The conditioning of the PER is another classical insect learning paradigm in insects, which has been successfully applied with triatomines (Vinauger et al. 2013). This well-characterized response used with bees, flies, butterflies and bumblebees constitutes a simple bioassay for investigating different facets of insect learning and memory. In the case of triatomines, the PER could be aversively conditioned in *Rhodnius prolixus* and revealed that these bugs can remember novel associations for at least 72 h (Vinauger et al. 2013). Furthermore, those experiments showed that they are only able to learn during the night hours, that is, in the temporal context when they display their daily activity (Vinauger and Lazzari 2015).

The successful application of different learning protocols in experiments with *Triatoma infestans* and *Rhodnius prolixus* also provided relevant information about how can the previous experience modify their responses to alarm and aggregation pheromones (Minoli et al. 2013; Mengoni et al. 2016), which are genetically determined, but modifiable by experience. Learning protocols also allowed demonstrating that kissing bugs can generalize and discriminate between different bitter compounds (Asparch et al. 2016), proving the utility of this approach for investigating basic sensory principles (Fig. 1).

6 Triatomine Chronobiology

A salient characteristic of the biology of triatomines is the marked temporal organization of their behaviour. It is known that selective pressures have acted to adjust the biting activity of blood-sucking insects to the time of the day when hosts are less active (reviewed by Barrozo et al. 2004b). However, the degree of synchronization of the various behavioural and physiological processes that have been proven to occur in triatomines is quite unusual. This temporal arrangement begins at a very early stage in the life of these bugs. Even 'once-in-a-lifetime' events in their biology are controlled by circadian clocks and not by the direct effect of the environment. First-instar larvae hatch from eggs in the early morning when the environmental relative humidity reaches a daily maximum. Nevertheless, this is not triggered by environmental changes but by an endogenous circadian clock (Lazzari 1991a; Schilman et al. 2009). The same temporal window is used for ecdysis across the various instars. Again, a circadian clock has been proven to control this process (Ampleford and Steel 1982). The temporal synchronization of egg hatching and ecdysis has an evident adaptive value that is probably related to the deleterious effect of low relative humidity on these processes. As indicated above, some triatomine species exhibit a marked xeropreference and this preference is not modified at egg laying activity nor for moulting (Guarneri et al. 2002; Roca and Lazzari 1994).

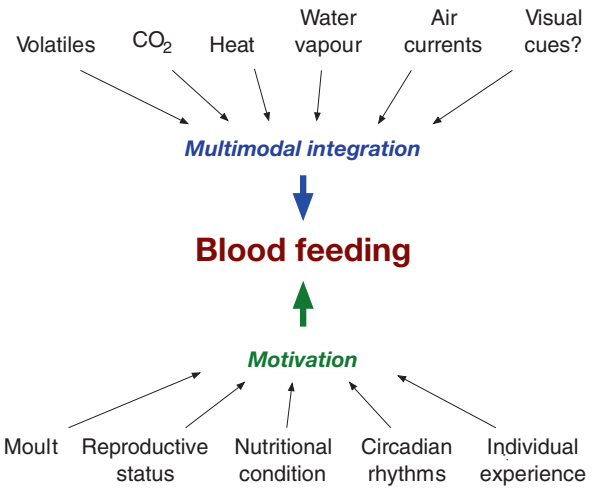

Fig. 1 Schematic representation of factors modulating host search in triatomines and other blood-sucking insects. The search for a host depends on external stimuli, helping the insect to find a potential blood source, and internal factors modulating its motivational state. The balance between inducing and inhibiting drivers will determine whether or not the insect will respond to the presence of a host, as well as the timing of these responses and the selection of the most appropriate host to feed on

Thus, we can conclude that triatomines exhibit a temporal hygropreference rather than a spatial one to adapt their needs at critical moments of their life. Instead of moving to humid places to perform humidity-sensitive activities, they perform them at a specific phase of the day that usually presents high humidity.

Even though triatomines are frequently described as nocturnal insects, their general, spontaneous activity can be categorized as bimodal, provided that it splits into two different temporal windows: one that encompasses the first hours after dusk and a second during the first hour after dawn, each controlled by a different internal oscillator (Lazzari 1992). These two peaks of activity comprise all the various activities displayed by these bugs, including the modulation of related sensory sensitivities. As a result, part of these processes take place during the first activity period, for example, host search, feeding, departure from refuges, egg laying and dispersion by flight, while others occur during the second activity peak as, for example, ecdysis, egg hatching, refuge search and bug aggregation (Barrozo et al. 2004a, b; Constantinou 1984) (Fig. 2).

The behavioural response to external stimuli is also rhythmically modulated as evidenced by the fact that the sensitivity of the insects is higher at the time in which a given cue or signal becomes biologically relevant. As an example, bug eyes become more sensitive to light during the night thus enhancing the negative phototactic response of bugs (Reisenman et al. 2002; Reisenman et al. 1998). The responsiveness to odours also varies in a very specific manner, as it is maximal to host odours during the nocturnal activity peak, but during the early morning peak in the

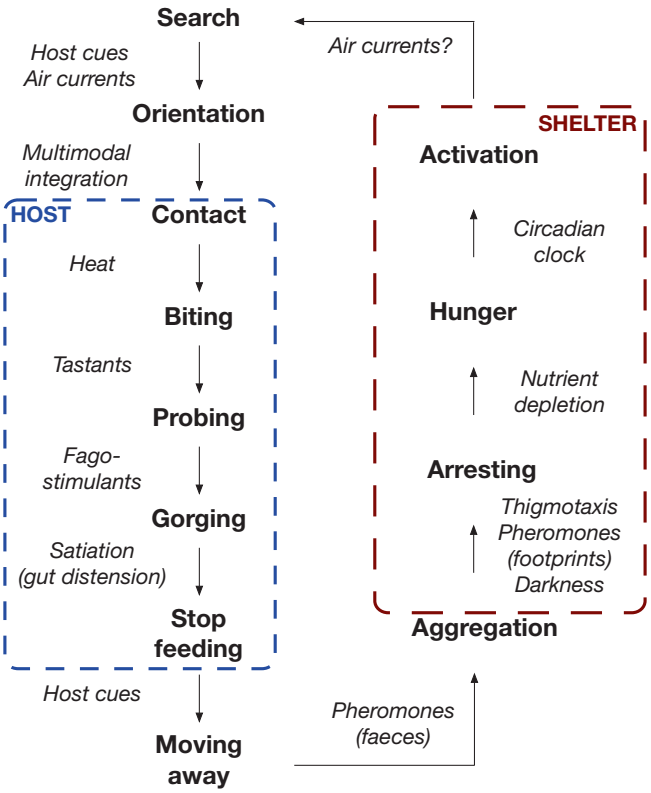

Fig. 2 Host and refuge search are two linked, but mutually excluding behaviours in triatomines. When the animal has consumed its nutritional reserves, it should go out from its refuge to look for a potential host. Once gorged, the bug looks for shelter. The figure synthesises the different steps and stimuli associated with these two activities, in the form of a loop that makes bugs alternating between food and shelter search

case of aggregation pheromones. These modulatory changes match the time of the day in which each olfactory stimulus has biological relevance, maximizing response efficiency (Barrozo et al. 2004a, b; Bodin et al. 2008).

A rhythmic change in the thermopreference of bugs has been described in several triatomines species. It causes bugs to expose themselves to relatively higher temperatures at the beginning of the night, before displaying the first activity peak. In the early photophase at the end of the second peak, the bugs return to cooler places. This rhythm is also controlled by an internal circadian clock (Lazzari 1991b; Minoli and Lazzari 2003). It is suggested that this thermopreference rhythm is intended to increase the body temperature of bugs and, in turn, their metabolic rate, just before exhibiting their regular daily activity. Conversely, both body temperature and metabolism would be reduced during resting periods.

In summary, many triatomine activities and processes are modulated in the form of daily rhythms. Most of them express at the individual level (activity, oviposition

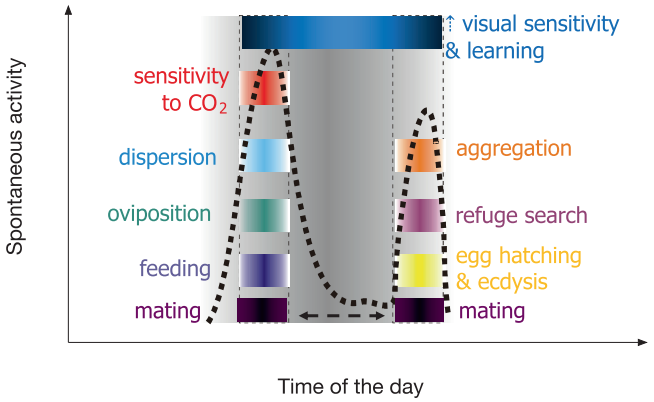

Fig. 3 The daily organisation of a kissing bug's life. Typically nocturnal, these insects distribute their different activities in two temporal windows, one at dusk and another at dawn. Bug sensitivity for stimuli such as odours and light is also modulated accordingly, in order to render them more sensitive and more prone to learn about their environment during the night hours

etc.), while others take the form of population rhythms (egg hatching and ecdysis). Interestingly, all daily rhythms have an endogenous basis and are controlled by circadian clocks with the exception of the modulation of aggregation pheromone sensitivity (Bodin et al. 2008) and the adaptation of ocelli to light (Lazzari et al. 2011). The precise temporal allocation of every sensitivity and action would be significant enough to cause the development of dedicated, specific clocks by natural selection. The fact that each activity has a particular temporal window is not just the result of its association with one of the two activity periods but it is a result of the temporal adjustment of that activity to a particularly adequate temporal context, for example, host resting period. The study of the chronobiology of triatomines also contributes to the understanding of the insect clock structure and the manner in which it coordinates different physiological processes (Steel and Vafopoulou 2006; Vafopoulou et al. 2007; Vafopoulou et al. 2010), revealing once more the value of these bugs as model systems to analyse fundamental biological questions.

One important consequence for researchers dealing with this strong temporal organization of triatomine physiology and behaviour is the conclusion that any process of interest must be studied in its appropriate temporal context. If the proper timing is not respected in studies of this nature, the results obtained may be biased, therefore masking the significant patterns (Lazzari et al. 2004) (Fig. 3).

7 Behavioural Manipulation

Controlling disease vectors by exploiting their behaviour requires a deep knowledge of their biology. Information such as the identity of relevant attractive cues, the profile of preferred shelters and environments, as well as the estimation of their

ability to escape from traps is potentially useful for the planning of sampling, detection and killing activities designed for controlling these insects. It is also critical that the stimuli used to attract the insects, as well as their presentation context (i.e. environment, timing), were biologically relevant and coherent to the insect, and not just a combination of all potentially attractive stimuli (pheromones, host odours, light); any association of stimuli must be significant for the insect. Here, 'biological context' means a clear message (i.e. host, refuge, partner), an adequate spatial setting (indoors, outdoors, ground, wall, roof) and timing that matches the proposed information to the insect needs (e.g. time for host search or for refuge localization).

In addition to light-traps employed for trapping nocturnal insects, whose attractivity principle is not fully understood, trapping triatomines and other blood-sucking insects is mostly based on the use of baits delivering host-associated cues. The most efficient is, as expected, the use of live hosts which usually consist of a mouse or baby chicken (Abad-Franch et al. 2000; Noireau et al. 1999, 2002). The use of live hosts as bait can sometimes be impractical due to the difficulty of providing them adequate care during extended field campaigns. Some alternative baits have been proposed and have already passed both laboratory and field testing. The simplest is using Baker's yeast cultures (a simple mixture of yeast, sugar and water) producing carbon dioxide and other volatiles that are attractive for kissing bugs (Guerenstein et al. 1995; Lorenzo et al. 1998, 1999a, b; Pedrini et al. 2009; Pires et al. 2000). In addition, a relatively simple combination of multimodal cues including heat, water vapour, carbon dioxide and short-chain fatty acids has been reported (Ryelandt et al. 2011). Both types of baits described above deliver compounds recognized to be attractive to many haematophagous arthropods and are useful for capturing not only blood-sucking bugs, but also mosquitoes and ticks (Ryelandt et al. 2011; Smallegange et al. 2005).

Artificial refuges operate in a different manner than baited traps. Rather than attracting the bugs, they provide convenient resting places. Due to the physical structure that induces bugs to enter and remain inside, they can even be used without chemical baits. Eventually, the bugs will produce 'their own bait' when their excrement, containing aggregation pheromones, starts to accumulate, attracting other bugs searching for shelters. Cardboard boxes simulating refuges have been used as part of the intra-domiciliary surveillance of Chagas disease vectors in endemic areas of Latin America (Gómez-Núñez 1965; Wisnivesky-Colli et al. 1987). Other refuge-like devices, composed of simple, resistant materials, have also proven to be effective for outdoor bug detection (Vazquez-Prokopec et al. 2002). An attractive perspective for the use of shelter-like devices is the incorporation of chemical lures into artificial refuges, as tested by Mota et al. (2014) and Forlani et al. (2015). Some devices combine attractive baits with killing or trapping tools (Pedrini et al. 2009; Rojas de Arias et al. 2012).

8 Perspectives and Research Needs

Our present knowledge allows identifying relevant aspects of the triatomine biology needing additional research efforts. Moreover, further investigation could provide important insight into the fundamental aspects of haematophagy and help in the development of novel control tools. The availability of the genome sequence of *Rhodnius prolixus* is an extraordinary opportunity to dig deeper into the genetic and molecular bases of haematophagous behaviour, confirming once more this species as one of the most useful and fascinating models available in insect science. I will mention here only three aspects that, in the opinion of the author, need to be prioritized.

Dispersion Dispersion is a key element for the spread of Chagas disease and for the reinfestation of treated houses (Abrahan et al. 2011). Several relevant questions remain to be answered, in particular, concerning the sensory ecology of long-distance displacements (e.g. the use of terrestrial and celestial navigation cues, orientation mechanisms).

Behavioural Impacts of Infection It is well known that many parasites modify the physiology and behaviour of vertebrate hosts and insect vectors to their own advantage (Lehane 2005; Schaub 2006). The behaviour of parasitized bugs is a major issue for which very restricted data are available. *Trypanosoma cruzi* infections seem to have physiological consequences for the insects (Vallejo et al. 2009; Fellet et al. 2014; Elliot et al. 2015), and it is highly probable that their behaviour is also affected. It has been shown that some key behaviours, like feeding and activity, differ between infected and non-infected bugs (Botto-Mahan et al. 2006; Marliere et al. 2015), yet the impact of infection on other behavioural traits needs to be further investigated, along with the underlying mechanisms (Takano-Lee and Edman 2002).

Behavioural Manipulation Synthetic pheromones could be used to interfere with bug communication in the sexual, alarm and aggregation contexts. Even though these alternatives appear as promissory options and despite the steadily advancing knowledge on this field, the use of synthetic pheromones for triatomine behavioural manipulation still requires further research efforts to be useful at an operational level.

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Features of Interaction Between Triatomines and Vertebrates Based on Bug Feeding Parameters

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Abstract Hematophagy is an unusual feeding strategy among arthropods occurring in <1.6% of phylum diversity. However, hematophagous arthropods possess great medical-veterinary relevance, as they act as vectors of important vertebrate diseases. Hematophagous species obtain blood from live vertebrates once they have successfully overcome key challenges, such as the penetration of mouthparts into the host epidermis, successful location of blood within the dermis, and pumping blood into the arthropod digestive tract. Despite a marked phylogenetic diversity among bloodsucking arthropods, adaptive convergences related to hematophagous behavior are observed, such as morphological adaptations of mouthparts, including structures selected for perforating, penetrating, and anchoring mouthparts in host skin, as well as the composition of saliva, which is rich in biomolecules capable of interfering with vertebrate physiology at the feeding site.

Triatomine bugs are temporary ectoparasites; their contact with their hosts is restricted to a blood meal. Blood is obtained directly from blood vessels in vertebrate skin. The following two physical sites of the triatomine–host interface are relevant during this process: a) the bug's "functional mouth" and host endothelium and b) the insect anterior midgut and host blood. In triatomines, most of the feeding time covers blood intake from host skin blood vessels to the insect anterior midgut. Blood intake is performed mainly through cibarial pump activity in these insects. The product of pumping frequency (F) and liquid volume ingested through each cibarial pump contraction (QLC) corresponds to the effective intake rate (EIR, mg/min) achieved by the insect during feeding. This is the triatomine feeding parameter that influences contact time with the host the most. The EIR of fifth instar nymphs fed on mouse abdominal skin can vary by more than 600% among triatomine spe-

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cies. The main parameter associated with differences in EIR between triatomine species, and among nymphal stages feeding in similar conditions, is QLC. Furthermore, cibarial pump frequency is the EIR component affected the most for a given species or development stage, by external factors such as host species (avian or mammal), feeding site characteristics (e.g., vessel diameter), and blood behavior within the midgut environment (e.g., coagulated/uncoagulated, aggregated/not aggregated). The likelihood that intestinal environmental factors affect feeding pump activity opens new perspectives for studying the impact of midgut colonization by different pathogens/microbiota on feeding performance, and/or the vectorial capacity of hematophagous arthropods.

Keywords Triatomines · Vertebrate host · Feeding parameters · Feeding site · Saliva · Insect anterior midgut

1 Initial Considerations

Hematophagy is an uncommon feeding behavior among arthropods, practiced by <1.6% of all ~1.21 million species described. It is believed that there are approximately only 14,500 hematophagous species distributed in the orders Diptera, Hemiptera, Siphonaptera, Lepidoptera, and Phthiraptera within the class Insecta. This strategy evolved solely in mites (~45,000 species) within the class Arachnida, where it is thought that less than 10% of species feed on blood (Ribeiro and Arca 2009; Mans 2011; Stork 2018). Therefore, the estimated number of blood-sucking arthropod[1] species is lower (<56%) than the ~33,000 species of major terrestrial vertebrate hosts. Since most hematophagous arthropods may be perceived, or even killed by the host during a blood meal, rapid feeding may be advantageous (Gillett 1969; Sant'Anna et al. 2001). Modifications of blood feeding performance in the vector appear to be common strategy found in many parasite–vector associations to enhance parasite circulation (Hurd 2003). In general, it is during hematophagy that pathogens circulate between the vertebrate host and their vectors. In addition, ingested blood may represent the only nutrient source for some arthropods (e.g., triatomines, ticks, lice, bed bugs) or as a nutritional stimulus for oocyte production (e.g., sandflies and mosquitoes). Therefore, differences in feeding performance directly affect vector competence and/or insect vector population dynamics.

In Hemiptera with sucking mouthparts, the presence of piercing elements and feeding pumps facilitated the emergence of different liquid diets. Although most hemipteran species (within the suborder Heteroptera) are phytophagous or predatory, obligatory hematophagy appears in three families (Cimicidae, Polyctenidae and Reduviidae). The largest number of hematophagous species (150) are within

[1] Hematophagous species of the class Crustacea, such as parasitic isopods of the family Gnathiidae, are not included.

the Reduviidae family, all of which were grouped into the subfamily Triatominae (Cobben 1978; Monteiro et al. 2018).

The topics covered in this chapter have been organized into two interrelated sections: the first one considers general aspects of hematophagy with an emphasis on triatomines, while the second, a compilation of published data on triatomine feeding behavior, aims to provide an understanding of how vectors and hosts interact at the feeding site, and how the midgut environment affects blood ingestion in hematophagous arthropods.

2 General View of Hematophagy

Hematophagy has arisen on at least 20 distinct occasions in the course of arthropod evolution. This promoted a series of independent, though functionally convergent physiological adaptations to solve common challenges relevant to hematophagous behavior, including a morphologically specialized feeding apparatus (Black and Kondratieff 2005) and various salivary biomolecules (Ribeiro and Francischetti 2003; Valenzuela 2004; Andrade et al. 2005; Ribeiro and Arca 2009; Arca and Ribeiro 2018) to enable feeding on blood from vertebrate host skin.

The skin of vertebrates is divided into an outermost layer called epidermis and two inner layers called dermis and hypodermis. The dermis is the thickest layer of the skin where blood vessels are located. However, only a fraction of the total skin area (<5%) is normally irrigated by blood vessels (Ribeiro, 1987). Blood is a tissue containing different types of cells, for example, red blood cells (RBCs), white blood cells (WBCs), and platelets or thrombocytes, and intercellular liquid (i.e., plasma) containing proteins, ions, and other molecules (Lewis 1996; Baskurt and Meiselman 2003).

Blood composition is relatively uniform among vertebrates with the exception of groups with nucleated red blood cells containing high levels of nucleic acids, such as birds, reptiles, and mammals of the family Camelidae. Whole human blood contains 80 g of water, 0.6 g of lipids, 0.08 g of carbohydrates, and 20.5 g of proteins per 100 mL (Lehane 2005). Thus, blood is composed of ~80% water and has a dry weight of ~97% protein.

Hematophagous species obtain blood from live vertebrates upon successfully overcoming key challenges, such as penetration of mouthparts into the host epidermis, location of blood in the host dermis, and pumping host blood into the arthropod digestive tract.

Despite morphological variations observed in mouthparts of hematophagous arthropod groups, it is possible to observe functional similarities, including structures selected for puncturing, penetrating, and anchoring, as well as sheath-like covering, a food canal, and a separated canal for saliva ejection owing to adaptive convergence to enable blood sucking (Krenn and Aspöck 2012). Insects possess a salivary canal that is independent of the food canal allowing them to simultaneously ingest blood and release saliva into host skin. In ticks, blood intake and saliva ejec-

tion occur alternately, as these arthropods use the food canal for both purposes (Lavoipierre and Riek 1955; Costa et al. 2016). Saliva is channeled through maxillary stylets only in fleas (Siphonaptera) and the order Hemiptera (Wenk 1953).

In triatomines, the proboscis is composed of a three-segment labium that encompasses a pair of mandibles and maxillae, both of which are long and needle-like. Mandible tips feature a row of teeth on their edges, whereas maxillae are thinner and have smoother structures (without teeth and bristles). Maxilla stylets articulate with each other to form two channels: a central food canal and a narrow alimentary canal (Lavoipierre et al. 1959). Triatomine mouthparts have unique features among Reduviidae bugs; their mandibles only have one row of strong teeth aligned along the midline, while their maxillary stylets lack bristles (Weirauch 2008). Such peculiar characteristics in relation to predatory members of Reduviidae can be morphological adaptations to hematophagous behavior in triatomines, as these allow a clean cut in the epidermis by the mandibles and less cellular destruction through smooth maxillae movements within the vertebrate host dermis.

After overcoming the epidermis barrier, hematophagous arthropods need to bring the alimentary canal opening (also called "functional mouth") into contact with blood. They use two basic mechanisms to achieve this, namely solenophagy ("vessel-feeding") and telmophagy ("pool feeding"), and these terms were proposed by Lavoipierre in 1965. In solenophagy, the alimentary canal opening is introduced directly into the blood vessel. Depending on the size of the blood vessel, it is possible to observe vibrations in its wall during blood ingestion by arthropods (Lavoipierre et al. 1959; Soares et al. 2014) (Fig. 1A). This feeding strategy is found in triatomines, mosquitoes, and lice (Lehane 2005).

Telmophagy consists of positioning the functional mouth in the skin region that contains blood from a vessel lacerated by the mechanical action of the arthropod's mouthparts. The formation of a feeding pool on mouse skin takes ~5% of total contact time (~ 40 min) in the soft tick *Ornithodoros rostratus*, with intense activity of the chelicerae and abundant salivation observed at this stage. Blood ingestion is characterized by a rhythmic variation in pool volume surrounding the tick's mouthparts (Costa et al. 2016) (Fig. 1A–C). Among all arthropods, the pool feeding strategy is present in sandflies, ticks, tabanids, ceratopogonids, tsetse flies, and blackflies (Lavoipierre and Riek 1955; Lehane 2005). Depending on feeding site conditions, certain vessel feeders may feed on blood extravasated from vessels, as reported in mosquitoes (Lavoipierre 1965).

It is the activity of the arthropod's mouthparts in search of blood that damages skin structures (cells and vessels) resulting in a release of or exposure to various host molecules in the invertebrate feeding site regardless of the feeding mechanism (solenophagy or telmophagy). Some of these molecules are mediators of host physiological responses (e.g., ADP, ATP, and collagen) that prevent blood loss (hemostasis) and promote tissue repair (inflammation).

Mammals have developed sophisticated mechanisms to limit blood loss after a vascular injury, a phenomenon known as hemostasis. This usually begins with a transient vasoconstriction episode followed by platelet adhesion/aggregation and blood coagulation (Ratnoff 1987). The injury of tissue (not only vascular), platelet

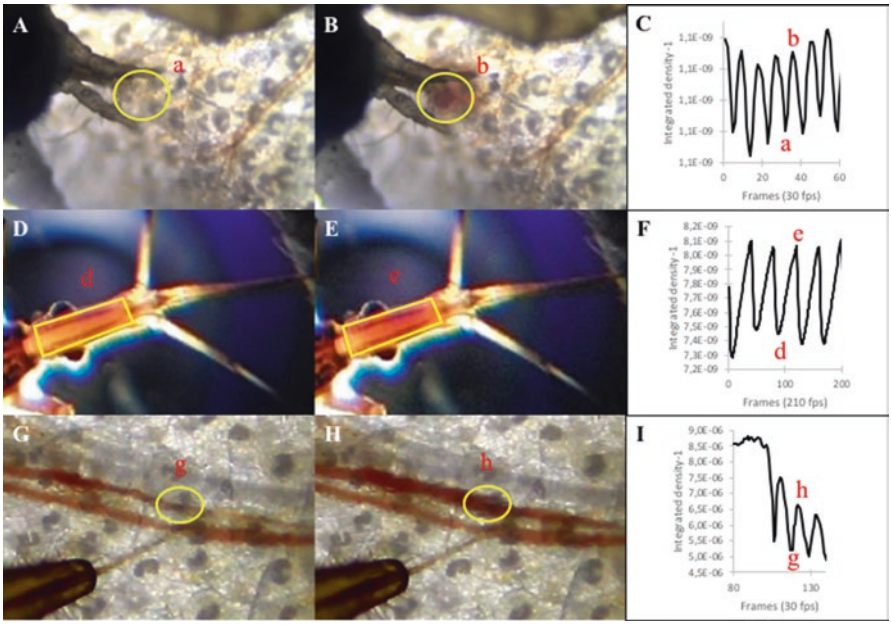

Fig. 1 Analysis of intravital images recorded during arthropod blood feeding on mouse skin A–B: Images of a 4th instar *Ornithodoros rostratus* feeding site, with the area around the feeding pool selected (marked by the yellow line) as minimal (a) or covered with blood (b). D–E: Images of a 5th instar *Rhodnius prolixus* head with the area around empty (d) or blood-filled (e) with the cibarial pump selected. G–H: Images of the feeding site of a 5th instar *Triatoma infestans*, with the area around the cannulated venule selected (marked by the yellow line) with minimal blood (g) or its lumen full of blood (h). C, F and I: The graphical representation of an estimate of the area occupied by blood within the selected sites in each frame over time. This calculation was performed using the Integrated Density function of ImageJ program (https://imagej.nih.gov/), according to Paim et al. (2017)

activation/aggregation, together with the activation of blood coagulation triggers the induction of inflammation, which is characterized by cardinal features such as rubor (redness) and tumor (swelling), as well as calor (heat) and dolor (pain). All of these involve changes in microcirculation: redness and heat reflect vasodilation, while pain is produced by the stimulation of nociceptors located in the inflamed tissue, which can be intensified by the swelling caused by increased vascular permeability (Ley 2008; Ribeiro and Francischetti 2003; Ribeiro and Arca 2009).

In addition, hemostasis and inflammatory reactions may be exacerbated by the development of immune responses (both innate and adaptive) against the arthropod's salivary antigens released during a blood meal (Ribeiro 1987, 1995). Therefore, during hematophagy, arthropods encounter some or all of these reactions, which hamper blood acquisition, such as by decreasing blood availability at the arthropod's feeding site (e.g., hemostasis reactions), or trigger defensive host behavior (e.g., reactions to itch and pain).

In contrast, insect saliva is rich in bioactive molecules capable of interfering with hemostasis, inflammation, and vertebrate host immunity, aiding the acquisition of a blood meal (Ribeiro and Francischetti 2003). Fleas and most blood feeding nematocerans (mosquitoes, sandflies, and blackflies) are estimated to have between 100 and 200 types of protein in their saliva; insects from the suborder Brachycera (tsetse flies and tabanids) have about 250–300, triatomines have >300, and ticks have more than 500 bioactive proteins to aid hematophagy (Francischetti et al. 2009; Arca and Ribeiro 2018). Such differences in salivary composition could be related to feeding type (vessel feeding or pool feeding) and blood meal duration (Arca and Ribeiro 2018).

Lipocalins are the most abundant and diverse family of proteins present in the saliva of triatomines and ticks. They have been shown to bind small molecules such as biogenic amines, nucleotides, and eicosanoids, which are effectors of the host's hemostasis and inflammation (Andersen and Ribeiro 2017).

In most hematophagous species, the pattern of salivation during different phases of feeding, as well as information on the saliva deposition site (whether it is inside or outside the vessels) within the host skin, is poorly known. In a study that described the bed bug (*Cimex lectularius*) feeding mechanism visualizing the insect's blood meal through the skin of a mouse ear, Dickerson and Lavoipierre (1959) commented: *"We looked particularly for the discharge of saliva, but we never observed the ejection of any fluid from the mouthparts, either during probing or whilst the insect was feeding from a blood-vessel."*

Salivation in *Rhodinus prolixus* begins at the moment of the bite, continues until the end of the blood meal (Friend and Smith 1971), and shows two distinct patterns. During the probing phase, salivation is abundant and constant, whereas during the engorgement phase, saliva is slowly released into the blood vessels in low frequency pulses (Soares et al. 2006; Sant'Anna et al. 2017). Part of the saliva released by the triatomine is ingested along with the diet, enabling the salivary biomolecules to act on blood stored in the insect midgut (Ribeiro and Garcia 1980; Soares et al. 2006).

After locating the blood, the insect needs to pump it from host blood vessels to the insect midgut, where the meal is initially stored. In liquid feeding insects, the cibarial and/or pharyngeal regions are modified to form a feeding pump. The walls of these regions are reinforced in these cases and normally have resilin (a rubber-like protein) deposits (Edwards 1983) linked to cibarial dilator muscles (extrinsic visceral muscles). Dilator muscles are antagonized by circular intrinsic muscles in pharyngeal pumps lacking intrinsic muscles (retractors), whereas muscle activity results from the elasticity of the muscle wall in cibarial pumps (Smith 1985; Chapman et al. 2013). The cibarial pump is well developed, whereas the pharyngeal pump is poorly developed in hematophagous Hemiptera. Both are well developed in female mosquitoes (Chapman et al. 2013).

Over the past decade, the integrated use of different 3D imaging and analysis techniques (particle image velocimetry [PIV] and synchrotron X-ray microscopic computed tomography) has yielded a detailed description of the coordinated operation of both the suction pumps (cibarial and pharyngeal) of the arthropod. Mosquitoes have two pumping patterns, which can produce a continuous ingestion flow through

multiple small suction motions, and a burst mode involving a single high-volume stroke (Kim et al. 2011; Lee et al. 2009; Kikuchi et al. 2018). However, the context in which mosquitoes shift their pumping pattern (of small or high-volume strokes) is unclear.

A recent study of *R. prolixus* synchrotron X-ray images showed that the frequency of the reduced pharyngeal pump matches that of the cibarial pump, although it makes inverted contraction-relaxation movements during fluid intake by the insect (Lahondère et al. 2017).

In contrast to bloodsucking insects and argasid ticks that normally spend a few minutes to <2 h on feeding, it takes several days for ixodid ticks to complete their blood meal on the host. The size of blood meal varies greatly between different groups of hematophagous arthropods. The gain in adult female body weight after a blood feed can reach ~110–140% in culicids, ~130% in cimicids, ~200–300% in triatomines and over 1000% in ixodids (Lehane 2005; Flynn and Kaufman 2015; Sant'Anna et al. 2017). The volume of blood ingested can be influenced by several factors such as the age, body size, nutritional status, and reproductive state of the arthropod. Under conditions where no physical restrictions to maintain blood pumping exist, triatomine and culicid blood meal sizes are regulated by the activation of abdominal stretch receptors, which signal the optimal blood meal volume to be ingested into the insect's midgut to the brain (Maddrell 1964; Gwadz 1969).

For temporary ectoparasites, visiting the host for a blood meal is very dangerous. Any appropriate strategy must include the maximization of nutrient intake rates, and the minimization of the host's contact time to overcome risks (Lehane 2005), while the role of feeding mechanics in determining constraints of such optimal strategies is often neglected (Daniel and Kingsolver 1983).

From a mechanical point of view, blood can be considered a solid–liquid suspension with cellular elements (primarily red blood cells) being the solid phase. Apparent blood viscosity depends on the shear forces present (since blood behaves like a non-Newtonian fluid), and it is determined by factors such as blood hematocrit (the volume percentage of RBC in blood), plasma viscosity, RBC aggregation, and RBC deformability. The viscosity of vertebrate blood decreases with hematocrit reduction and with an increase in temperature and shear rates, and the latter results from the movement of blood cells within vessels. Red blood cells tend to aggregate and adhere to each other (erythrocyte aggregation) with an intensity varying between species. The less aggregated the red blood cells, the more easily the particle suspension can move along a blood vessel (Kingsolver and Daniel 1995; Baskurt and Meiselman 2003).

Few models have been proposed to describe blood feeding mechanics to date, with those put forward mainly considering physical characteristics of the proboscis (mainly the diameter/length of the initial section of the alimentary canal), and the difference in pressure generated by the insect's feeding pumps (Kornev et al. 2017). An analysis of these models based on the description of blood flow through narrow tubes suggested that the biomechanics of blood feeding can be explained by the Hagen–Poiseuille equation for Newtonian fluids (Bennet-Clark 1963a, b; Daniel and Kingsolver 1983; Lehane 2005). According to Kingsolver and Daniel (1995),

parameters such as negative pressures produced by the cibarial pump, insect food canal dimensions, blood viscosity, the host's red blood cell size, and deformation capacity can all influence blood ingestion rate.

Although there are important differences in blood meal parameters (size, duration, and periodicity) between blood feeding arthropod groups, a similar sequential pattern of events is observed during feeding by most bloodsucking insects and argasid ticks. Thus, based on the movement of mouthparts and/or feeding pump activity, the arthropod's blood feeding process can be divided into at least four basic steps: bite, probing, engorgement, and interruption. Bite corresponds to the moment when mouthparts penetrate the outer part of the skin. Probing occurs when the arthropod actively moves its mouthparts searching for blood in the host dermis. Probing is preceded by the bite, but it can also occur at any time during the feeding process without the need for the arthropod to remove mouthparts from the host skin. Engorgement is the period in which the arthropod effectively ingests blood with feeding pumps in operation. Interruption is the moment when blood intake is stopped without the withdrawal of mouthparts from the host skin, but with feeding pumps inactive (in the case of argasid ticks, apparently there is no saliva ejection). With the exception of interruption, all events appear at least once during blood feeding, although they can happen several times during the blood meal.

3 Triatomine Blood Feeding Characteristics

Triatomine bugs are temporary ectoparasites, and their contact with their hosts, both for immature instars and adults, is restricted to a blood meal. Feeding motivation is rhythmically modulated, with individuals more likely to feed during the first hours of the night (Lazzari et al. 2013). These insects are vessel feeders obtaining their blood meals directly from blood vessels (venules or arterioles) of their vertebrate hosts. After the bug pierces host skin, a probing period, characterized by rapid whip-like intradermal movements of the maxillae, can be observed (Lavoipierre et al. 1959). During this initial phase, *R. prolixus* samples the liquid around its maxillae periodically, analyzing its composition to detect the presence of phagostimulants (cues of blood presence—ATP and ADP nucleotides). Eight gustatory sensillae located in the cibarium are used for this purpose (Friend and Smith 1971; Pontes et al. 2016). Once a suitable vessel is found, probing ceases and the bug engorges with blood (Lavoipierre et al. 1959). The ingestion of blood through the food canal is aided by the cibarial pump, the filling of which is accomplished by extending muscles from the dorsal wall of the head, while emptying is achieved by returning the piston under the force of the elastic ligament (Bennet-Clark 1963a, b).

Fifth instar nymphs of *R. prolixus* can achieve high ingestion rates (~20 mg/min at a flow rate of ~0.33 μL s^{-1}) despite a narrow food canal at the apex (8–10 μm in diameter) (Bennet-Clark 1963a, b). Conservative calculations suggest that the cibarial pump of *R. prolixus* is able to generate pressure differences of 1–2 atm during feeding (Kingsolver and Daniel 1995).

At the beginning of the blood meal, the abdominal cuticle of *R. prolixus* becomes less rigid (plasticization) allowing the abdomen to dilate, thereby accommodating a proportionately large amount of food in the anterior midgut (Bennet-Clark 1962; Maddrell 1966). Cuticle plasticization, as several other feeding-related physiological responses, are under neural control mediated by hormones secreted into the hemolymph, occurring as soon as the insect touches/bites host skin (Maddrell 1966; Reynolds 1974; Ianowski et al. 1998).

Orchard (2006) argued that the neurohormone serotonin (5-HT) released by nerve terminations coordinates the steps of a blood meal in *R. prolixus*, acting as an effector, additive or synergic agent in several feeding-related events such as: a) salivation; b) water and ion transport from the anterior midgut to the hemolymph, and the concentration of ingested blood cells and plasma proteins; c) cuticle plasticization; d) the elevation of dorsal vessel frequency, which is important for increased hemolymph circulation to hormone-target tissues (potential energy supply to the cibarial pump), and for insect thermoregulation (see Lahondère et al. 2017); e) urine formation and post-prandial diuresis (Orchard 2006).

The technique (electrical penetration graph [EPG]) to study feeding parameters in triatomine bugs was standardized by Smith and Friend (1970), registering changes in electrical resistance between bugs and their feeding source. This methodology was modified by Smith (1979) and Guarneri et al. (2000) for recording signals generated by the cibarial pump's musculature, similar to an electromyogram (Araujo et al. 2011).

Combined analysis using electrical signal profiles and intravital microscopy (targeting cibarial pump activity and vessel wall movements, see Fig. 1) obtained during the insect's blood meal combined the information from both methodologies to describe different phases of triatomine blood feeding in detail (Soares et al. 2014) (see details in Table 1).

Total contact time (TCT) of bugs with their feeding source (corresponding to "feeding time") is briefly divided into two parts: the ingestive (IP) and non-ingestive periods (NIP). The former comprises the sum of all engorgement periods (with the cibarial pump in activity), while the latter is the sum of probing and interruption events that occur during blood feeding (cibarial pump downtime). Using the bug's weight gain (WG) upon feeding (corresponding to "blood meal size"), two distinct ingestion rates can be calculated: the total ingestion rate (TIR), related to TCT (TIR = WG/TCT), and the effective ingestion rate (EIR), related to IP (EIR = WG/IP). The effective ingestion rate can also be obtained by multiplying the frequency of pumping (F) by the quantity of liquid ingested by each cibarial pump contraction (corresponding to "stroke volume"). Thus, TCT can be calculated from EIR ($TCT = [WG \times (F \times QLC)^{-1}] + NIP$). This equation allows for a good understanding of the feeding process through relationships among different feeding parameters.

To evaluate host influence (host species or feeding site) or the impact of insect characteristics (feeding apparatus, developmental stage, and salivary gene) on feeding parameters, we compiled electromyogram feeding behavioral data for seven triatomine species (three *Triatoma* and four *Rhodnius*), four species of host (three mammals and one avian) with different feeding site characteristics, with hosts treated with anti-hemostatic drugs (heparin) or insects injected with dsRNA (RNAi) for salivary genes (Table 1).

Table 1 Blood-feeding parameters (see the definitions below) of different triatomines (species, stages, treatments) fed on live hosts

Species[a]	Stage	TCT (min)	IP %	NIP %	WG (mg)	EIR (mg/min)	F (Hz)	QLC (ηL)	Host[b]	Site[c]	Refs[d]	Line
T. brasiliensis	1st	18.3	89.0	11.0	6.5	0.4	2.8	2.7	H	FS	3	1
T. brasiliensis	2nd	18.7	91.4	8.6	24.0	1.4	3.3	7.1	H	FS	3	2
T. brasiliensis	3rd	25.5	91.0	9.0	46.3	2.0	2.6	12.6	H	FS	3	3
T. brasiliensis	4th	23.5	96.2	3.8	85.9	3.8	2.4	26.9	H	FS	3	4
T. brasiliensis	5th	33.9	93.8	6.2	359.3	11.3	3.0	62.8	H	FS	3	5
T. brasiliensis	5th	20.2	76.6	23.4	264.7	17.1	3.9	71.4	P	BS	2	6
T. brasiliensis	5th	29.3	70.8	29.2	271.8	13.1	3.0	70.5	M	AS	2	7
*T. brasiliensis**	5th	34.0	100.0	0.0	384.2	11.3	3.0	62.8	H	FS	4	8
*T. brasiliensis**	5th	15.7	72.6	27.4	70.7	6.2	2.1	49.2	Tl	AS	4	9
*T. brasiliensis***	5th	37.6	100.0	0.0	413.6	11.0	2.7	66.3	H	FS	4	10
*T. brasiliensis***	5th	23.2	77.6	22.4	127.6	7.1	2.3	51.4	Tl	AS	4	11
T. infestans	5th	9.9	90.2	9.8	250.0	28.0	4.3	109.0	P	BS	2	12
T. infestans	5th	14.2	64.8	35.2	195.9	21.3	3.4	102.1	M	AS	2	13
T. pseudomaculata	5th	34.5	88.0	12.0	157.9	5.2	3.5	26.0	P	BS	2	14
T. pseudomaculata	5th	36.2	77.5	22.5	115.0	4.1	3.0	23.6	M	AS	2	15
R. nasutus	5th	17.8	63.4	36.6	123.0	10.9	4.3	42.4	P	BS	5	16
R. nasutus	5th	17.6	73.7	26.3	129.0	9.9	4.0	41.3	M	AS	5	17
R. neglectus	5th	25.2	84.3	15.7	140.0	6.6	3.6	30.4	P	BS	5	18
R. neglectus	5th	46.5	73.5	26.5	120.0	3.5	1.9	30.5	M	AS	5	19
R. prolixus	5th	14.6	84.3	15.7	262.0	21.3	6.0	59.1	P	BS	5	20
R. prolixus	5th	24.6	69.8	30.2	260.0	15.1	4.8	52.5	M	AS	5	21
*R. prolixus****	5th	20.7	93.2	6.8	237.0	12.8	3.7	57.7	M	DS	1	22
*R. prolixus****	5th	17.4	100.0	0.0	270.0	16.0	4.7	56.7	M	TBV	1	23
R. prolixus	5th	43.9	84.3	15.7	229.0	8.6	2.7	53.1	M	DS	1	24
R. prolixus	5th	18.1	100.0	0.0	242.0	14.8	4.5	54.8	M	TBV	1	25

Species[a]	Stage	TCT (min)	IP %	NIP %	WG (mg)	EIR (mg/min)	F (Hz)	QLC (ηL)	Host[b]	Site[c]	Refs[d]	Line
R. robustus	5th	23.7	68.7	31.3	258.0	15.9	4.4	60.0	P	BS	5	26
R. robustus	5th	37.9	59.7	40.3	232.0	10.2	2.7	62.9	M	AS	5	27
T. brasiliensis	Male	17.2	81.4	18.6	145.3	10.4	2.5	67.9	H	FS	3	28
T. brasiliensis	Female	21.1	79.6	20.4	228.1	13.6	2.5	88.9	H	FS	3	29

Feeding parameters definition:
Total contact time [TCT (min)] is defined as the total time which the mouthparts of the bugs remain into the host skin.
Ingestive period [IP (min)] comprises the sum of all engorgement periods with the cibarial pump in activity
Non-ingestive period [NIP (min)] is defined as the period when insects are not pumping, thus comprising probing plus any interruption events
Weight gain [WG (mg)], is calculated by the weight of the insect after meal minus its initial weight (before feeding)
Total intake rate [TIR (mg/min)] is calculated by the weight gain divided by total contact time (TIR = WG/TCT)
Effective intake rate [EIR (mg/min)] is calculated by the weight gain divided by the ingestive period (EIR = WG/IP)
Frequency [F (Hz)] is the total number of cibarial pump contractions divided by the ingestive period (IP)
Quantity of liquid ingested by cibarial pump contraction [QLC (ηl)] is obtained by dividing the weight gain by the total number of cibarial pump contractions during the feeding process, considering blood densities as 1 mg/ml
[a]*domestic population; **sylvatic population; ***Nitrophorins$_{(1-4)}$ knockdown
[b]H: human; M: mouse; P: Pigeon; Ti: *Thrichomys laurentius*
[c]AS: Abdominal skin; BS: Breast skin; DS: Dorsal skin; FS = Forearm skin; TBV: Tail base vein
[d]1: Araujo et al. (2009a, b, c); 2: Guarneri et al. (2000); 3: Guarneri et al. (2003); 4: Guarneri et al. (2011); 5: Sant'Anna et al. (2001)

Table 1 provides an overview of feeding parameters presented by triatomines fed on live hosts, where a large variation between species is observed.

4 Total Contact Time

An important factor influencing the interaction between hematophagous arthropods and their hosts is TCT, as defensive host behavior may reduce blood feeding success or even kill the insect. When triatomines feed on unanesthetized hosts, the reduction in blood meal size due to an increase in insect density is modulated by the host's perception of the bugs resulting in shorter feeding times (Schofield 1994; Pereira et al. 1995, 1998). Therefore, species with higher blood ingestion rates possess a better capacity to exploit blood resources from available hosts inside human dwellings, thereby reaching higher population densities. A high feeding performance observed for both *T. infestans* and *R. prolixus* allowed these species to spread and become the most important vectors of *Trypanosoma cruzi* in South and Central America (Sant'Anna et al. 2001; Pereira et al. 2006).

Smith (1979) showed the effect of diet viscosity on *R. prolixus* feeding parameters when bugs were fed on an artificial feeder. He observed that an elicited increase in diet viscosity (0.8 to 6.5 cP) increased TCT by reducing TIR (~68%) and WG (~33%) in fifth instar nymphs. This TIR decline was due to a decrease in both F (~48%) and QLC (~38%).

Notably, TIR and EIR are similar when NIP (i.e., probing and interruption events during engorgement periods) is short, as observed with *Rhodnius* species when fed on an artificial feeder (Friend and Smith 1971; Smith 1979; Sant'Anna et al. 2001). However, this is not the case with the *Triatoma* species studied. They present frequent interruptions and partial engorgement, suggesting that they may need other stimuli to keep the cibarial pump functioning when feeding outside living hosts (Lazzari and Nuñez 1989; Guarneri et al. 2000). Therefore, it is desirable, when possible, to use EIR, as this parameter reflects only IP.

Firstly, data from seven triatomine species (four *Rhodnius* and three *Triatoma*) from the same developmental stage (fifth instar nymphs) were analyzed under similar experimental conditions using two hosts (pigeon and mouse) to evaluate how TCT behaves when bugs feed on live hosts, where all species were able to obtain similar WG values (Table 1. Lines: 6-7, 12-21 and 26-27). A comparison of these species showed a large variation in TCT (9.9–46.5 min) regardless of host species. Pearson's correlation coefficients of the feeding parameters of these species obtained indicated, that TCT mainly correlated with EIR (-0.81; $p < 0.05$), and that no significant correlation was found between TCT and WG (-0.35; n.s.) (Table 2). In turn, the difference in EIR (F × QLC) between species was more influenced by QLC (0.90; $p < 0.05$) than the frequency of the cibarial pump (0.60; $p < 0.05$) (Table 2).

Contrastingly, TCT for different nymphal stages of *T. brasiliensis* fed on human hosts ranged from 18 to 34 min (Table 1. Lines: 1–5). In contrast to that observed in fifth instar nymphs of different species, variation of EIR values among *T. brasiliensis*

Table 2 Pearson correlation coefficients of feeding parameters of seven triatomine species fed on pigeon and mouse[1]

	TCT (min)	WG (mg)	EIR (mg/min)	F (Hz)	QLC (nL)
TCT (min)	1				
WG (mg)	−0.346[b]	1			
EIR (mg/min)	−0.807[a]	0.712[a]	1		
F (Hz)	−0.747[a]	0.433[b]	0.598[a]	1	
QLC (nL)	−0.619[a]	0.660[a]	0.902[a]	0.214[b]	1

For variable definitions, see Table 1
[1]Data analyzed are shown in lines 6-7, 12-21 and 26-27 of Table 1
[a]$p < 0.05$ data
[b]not significant ($p > 0.05$)

Table 3 Pearson correlation coefficients of feeding parameters of *T. brasiliensis* nymphal stages fed on human hosts[1]

	TCT (min)	WG (mg)	EIR (mg/min)	F (Hz)	QLC (nL)
TCT (min)	1.00				
WG (mg)	0.93[a]	1.00			
EIR (mg/min)	0.93[a]	1.00[a]	1.00		
F (Hz)	−0.09[b]	0.15[b]	0.11[b]	1.00	
QLC (nL)	0.93[a]	0.98[a]	0.99[a]	0.02[b]	1.00

Variable definitions are used. See Table 1
[1]Data analyzed are in lines 1–5 of Table 1
[a]$p < 0.05$
[b]not significant ($p > 0.05$)

nymphal stages was similar to that between EIR (0.93; $p < 0.05$) and WG (0.93; $p < 0.05$) (Table 3). This difference can be explained by the fact that the anterior intestine and the cibarial pump, even though not in equal proportion, follow body growth during insect development. Values of WG for nymphal stages in *Triatoma* species was approximately 5 × of pre-feeding weight (Sant'Anna et al. 2017). Cibarial pump contraction is among the main factors responsible for EIR variation observed during the post-embryonic development of triatomines (0.99; $p < 0.05$). However, no significant correlation was found between EIR and F (0.11; n.s.) (Table 3). In *T. brasiliensis* adults, males and females presented marked sexual dimorphism regarding QLC. Females show better feeding performance compared to males, because they have higher QLC (Guarneri et al. 2000) (Table 1. Lines: 28-29).

In all of the triatomine-host associations analyzed above, most of the contact time was spent on the process of blood ingestion by cibarial pump action (ingestive period), corresponding to an average ~ 80% of TCT. Although the non-ingestive period (NIP) generally represents less than 20% of TCT, it is a critical phase, as it includes the initial probing step following the bite, corresponding to the host's most perceived feeding period (Schofield et al. 1986). Interruptions during IP are usually followed by a "secondary probing" in triatomines, even when the insect does not remove mouthparts from host skin.

Table 4 Pearson's correlation coefficients of the feeding parameter ratio (pigeon/mouse) obtained by feeding each triatomine species on both hosts[1]

	TCT (min)	WG (mg)	EIR (mg/min)	F (Hz)	QLC (nL)
TCT (min)	1.00				
WG (mg)	0.11[b]	1.00			
EIR (mg/min)	−0.79[a]	0.16[b]	1.00		
F (Hz)	−0.74[b]	0.10[b]	0.97[a]	1.00	
QLC (nL)	0.24[b]	0.26[b]	−0.41[b]	−0.62[b]	1.00

For variable definitions, see Table 1

[1]The data analyzed were obtained by dividing respective feeding parameters of each line, as follows: 6/7; 12/13; 14/15; 16/17; 18/19; 20/21 and 26/27 of Table 1

[a]$p < 0.05$

[b]not significant ($p > 0.05$)

5 Birds Versus Mammal Hosts

A comparison of feeding parameters obtained in the two hosts (pigeon and mouse) showed profound differences among triatomine species (Table 1). For example, TCT spent by *R. nasutus* on obtaining similar WG on mouse was almost equal to that spent on pigeon, while it was ~85% higher in *R. neglectus* (Table 1. Lines: 16-17 and 18-19). Interestingly, these two species belong to the same species complex (*R. prolixus* complex) and are phylogenetically very close.

Despite the differences found, all species presented better feeding performance in pigeons except for *R. nasutus*, evidenced by an average TCT decrease of ~30% and by an increase of 27% to 87% in EIR. The analysis of feeding parameter ratio (pigeon/mouse) obtained by feeding triatomine species on both hosts showed that the difference in TCT between the two hosts was more correlated with EIR (− 0.8; $p < 0.05$) and F (−0.7; $p = 0{,}056$) than the other parameters (Table 4). Although QLC is the main parameter associated with the differences in EIR found between species, and among nymphal stages (see above), the impact of host change (pigeon × mouse) in EIR in the same species is more related to F variation (0.97; $p < 0.05$), suggesting that cibarial pump frequency is the main indicator of impacts of external factors, such as different host species on insect feeding performance (Table 4).

These results demonstrate that most triatomine species show better feeding performance (higher EIR/F) when they take a blood meal from pigeons than from mice.

6 Saliva and Salivation During Blood Feeding

Ribeiro and Garcia (1981) were the first to show that triatomine saliva reduces probing and feeding times. They showed that salivectomized *R. prolixus* specimens fed equally well on blood presented in an artificial feeder (without host hemostasis/inflammation responses), whereas they fed slower in rabbits in comparison to sham-operated controls (Ribeiro and Garcia 1981).

However, marked differences in expressed molecules and salivary gland morphology exist within the subfamily Triatominae, observed particularly between the Rhodniini and Triatomini tribes (Ribeiro et al. 1998; Lacombe 1999).

Although nitrophorins (nitric oxide [NO] carrier hemeproteins) are the most abundant salivary lipocalins in the genus *Rhodnius*, this family of lipocalins does not occur in species of the Triatomini tribe (Ribeiro et al. 2012). In contrast, only the sialotranscriptomes of Triatomini species identified secreted protease sequences (Ribeiro et al. 2012).

Nitrophorins are responsible for the characteristic red coloration of Rhodniini salivary glands, in contrast to the colorless glands of Triatomini. Besides presenting anti-coagulant and anti-histaminic activities, *Rhodnius* salivary nitrophorins release NO into host skin, a potent vasodilator and anti-platelet aggregation agent (Champagne et al. 1995). Interestingly, only *Cimex* species (Cimicide) have NO as a vasodilator molecule among hematophagous Hemiptera apart from Rhodniini species, using completely different nitrophorins to carry this unstable gas (Ribeiro et al. 2012). Although NO elicits a potent and transient relaxation of vascular smooth muscles, long-term vasodilation is observed at an *R. prolixus* feeding site even after the end of the insect's feeding. One possible explanation for this long-lasting effect of saliva is that part of the NO released by nitrophorins binds to host proteins such as albumin, forming S-nitroso-proteins (S-nitroso-albumin). These in turn slowly release NO ensuring long-term vasodilation in host skin, ultimately enabling efficient hematophagy for longer periods of time (Paim et al. 2017).

Differences found in the composition of bioactive salivary molecules (such as NO, nitrophorins, and proteases) between the Rhodniini and Triatomini tribes will reflect the biological activity of their saliva. Comparative studies have shown quantitative and/or qualitative differences in triatomine saliva in properties such as anticoagulant, antiplatelet aggregation, vasodilatory, proteolytic, and inhibitory of the classical complement pathway (Pereira et al. 1996; Ribeiro et al. 1998; Amino et al. 2001; Cavalcante et al. 2003; Barros et al. 2009).

Comparisons of salivary gland morphology also show differences between species of the two tribes, in which salivary glands of Triatomini species consist of three lobes (D1, D2, and D3), whereas the D3 lobe is absent in Rhodniini species (Lacombe 1999).

The impact of salivary molecules on triatomine feeding parameters has been demonstrated in recent studies using knockdown insects (RNAi). While most knockdown *R. prolixus* specimens for salivary nitrophorins$_{(1\text{-}4)}$ probed 2–20 times before starting the engorgement phase in the dorsal surface of mouse skin, all insects from the control group presented only a single probing activity. The rate of effective ingestion was also affected by reduced nitrophorin expression by lowering pumping frequency in bugs feeding on dorsal mouse skin (Araujo et al. 2009a). Interestingly, the negative impact of silencing salivary nitrophorins on feeding performance was not observed, when the insects probed into a large mouse tail vessel (Table 1, Lines: 22-23), reiterating the importance of endothelial stress at the insect feeding site in triggering host hemostasis and inflammatory responses, as discussed here.

Triatomines feature an interesting mechanism regulating the release of saliva at their feeding site. The salivary pump of *R. prolixus* works intermittently during IP and has a low frequency (~0.5 Hz) compared with the cibarial pump (~ 4.5 Hz) when feeding on mouse skin. This large difference in frequencies implies that during periods when the insect is effectively ingesting blood, most of the egested saliva is ingested. However, at times when F is too low or blood pumping is interrupted, the quantity of released saliva increases within the canulated vessel. This mechanism modulates the quantity of saliva deposited into microcirculation, minimizing host immune response against salivary antigens (Soares et al. 2006).

Several substances aiding hematophagy have been described from triatomine saliva, including anticoagulants, vasodilators, antihistamines, sodium channel blockers, as well as of platelet aggregation inhibitors induced by ADP, thrombin, serotonin, arachidonic acid, platelet-activating factor (PAF), epinephrine, and norepinephrine. In addition to these substances, others such as compounds with immunosuppressive activity, a sialidase enzyme, and a pore-forming protein have also been described, as well as anti-complement activity aiding the feeding process, all reducing immune and inflammatory responses of the vertebrate host (Ribeiro 1995; Andrade et al. 2005; Ribeiro and Arca 2009; Ribeiro et al. 2012).

7 Triatomine–Host Interface

The host–parasite interface is the site of molecular exchange between the two organisms, operating in a two-way path where nutrients from the host enter the parasite and molecules from the parasite (e.g., metabolic wastes) enter the host. A detailed representation of this interface was described using a few models, mainly intracellular parasites, which have revealed complex interactions taking place (Trager 1986).

As shown here, triatomine bugs are temporary ectoparasites with most of their feeding time (TCT) used for pumping blood (IP) from host skin blood vessels to the insect anterior midgut. Therefore, the functional interface with the host involves both the blood feeding site and the insect midgut in this group.

7.1 Triatomine–Host Endothelium

As triatomines are "vessel feeders," the most enduring connection within the triatomine–host interface at the feeding site is established between the insect maxillae tips ("functional mouth"), with the release of saliva on one side and the vertebrate's vascular endothelium at the other side.

Vessel diameter decreases immediately upon the introduction of triatomine mouthparts, and visible pulsation promptly begins evidencing the onset of IP. The intensity of vessel wall movements varies considerably. Sometimes they are imperceptible, while other sometimes so accentuated that they can be easily observed

(Lavoipierre et al. 1959). Therefore, IP is preceded by vessel wall injury allowing the subendothelial matrix to be exposed to blood components, whereas cibarial pump operation in the insect causes large local oscillations in blood flow (wall shear stress) in the cannulated vessels.

The endothelium is a single layer of cells lining the lumen of vertebrate blood vessels. Mammalian endothelial cells play a crucial role in maintaining a balance between the activation and inhibition of the hemostatic system and inflammation. Under normal conditions, mammalian endothelial cells have a non-thrombogenic surface that does not support platelet adhesion or fibrin generation. They produce and release powerful soluble platelet activation inhibitors, nitric oxide, and prostacyclin. They also express ADPases (CD39) and thrombin inhibitors (i.e., thrombomodulin), which in turn rapidly metabolize two major platelet agonists, ADP and thrombin, respectively. This antithrombotic environment, however, may change rapidly after endothelium activation by vascular injury, or even in response to changes in fluid shear stress. The activated endothelium (i.e., a procoagulant environment) can actively facilitate fibrin generation and the recruitment of platelets and leukocytes through tissue factor expression, exposing ligands such as P-selectin and vWf through the synthesis of pro-inflammatory mediators (Nesbitt et al. 2006; Watson 2009).

Therefore, endothelial cells trigger hemostatic and inflammatory reactions once activated. These host responses may explain the increase in vascular permeability, platelet aggregation, and rolling/adherent leukocytes in the venular endothelium adjacent to the *R. prolixus* feeding site, as observed by an intravital microscopy study (Soares et al. 2014). It can also explain the accentuated accumulation of leukocytes around the region where *R. prolixus* maxilla are inserted into the vessel, as observed by histological techniques (Lavoipierre et al. 1959).

When triatomines take blood from host skin, mainly of mammals (mice), cibarial pump frequency does not remain constant throughout the feeding, but shows a tendency to decrease in its final period (Sant'Anna et al. 2001). The analysis of a collation of images of the cibarial pump in the head of *R. prolixus* (Fig. 1D–F), or of vessel wall movements (Fig. 1G–I), together with electromyographic records, allowed us to infer that changes in F observed during insect feeding are mainly due to variations in pump filling time (Soares et al. 2014).

Endothelial activation at the bug feeding site can generate a procoagulant environment suitable for platelet aggregation and thrombin generation, thereby changing rheological characteristics of blood. The blood clotting process basically consists of a transition from fluidic blood to a solid clot. Once the blood coagulation cascade has been initiated, a rapid increase in blood viscosity occurs (Puckett et al. 2005; Ranucci et al. 2014). It is known that an increase in diet viscosity leads to a decline in cibarial pump frequency (Smith 1979). Thus, it is plausible that an increase in blood viscosity at the feeding site could explain the increase in pump filling time as observed during *R. prolixus* blood meal on mouse skin (Soares et al. 2014).

Furthermore, given that the opening of the feeding channel is narrow (8–10 μm), the formation of microaggregates of platelets at the feeding site could increase the friction of food along the channel wall, which could also prolong the time of pump filling.

Within this context, it is expected that the smaller the vessel diameter of the vertebrate host, the greater the mechanical stress caused by insect cibarial pump activity (i.e., high amplitude of vessel wall movements and strong oscillations of flow). This could explain the fact that triatomines had greater difficulty in feeding from small vessels in mouse dorsal skin (Ø ~ 20–40 μm) compared with a large lateral vein (Ø > 500 μm) of the tail base (Table 1. Lines: 24-25) (Araujo et al. 2009a).

Following this logic, the best feeding performance was achieved in triatomines that fed on pigeon blood (>EIR/F) in comparison to mice, which might be related to differences between bird and mammal hemostasis. Although basic hemostatic mechanisms are conserved among vertebrates, marked differences among different vertebrate hosts exist. For example, the intrinsic coagulation pathway is a highly essential hemostatic mechanism in mammals, but less important in birds (Lewis 1996).

However, this greater difficulty for triatomines, especially in maintaining F during blood feeding when changing pigeons to mice, must be more related to differences found between platelets and thrombocytes, considering the relevant role of platelets in the coordination of hemostatic and inflammatory responses in mammals.

Thrombocytes in birds perform a similar function to platelets, but spread and/or aggregate less efficiently upon ADP, collagen, ristocetin, arachidonic acid addition, or thrombin exposure than those of mammals (Lewis 1996; Schmaier et al. 2011). Avian thrombocytes express lower levels of integrin α2bβ3 that plays an important role in aggregation (Schmaier et al. 2011), and do not have the cytoplasmic GP1b-alpha domain that is important for cytoskeletal rearrangement required for changing platelet shape (Ribeiro et al. 2015). These features make thrombocytes slower to induce blood clots to control bleeding, and their aggregates are less resistant to high shear forces in fluid than platelets (Schmaier et al. 2011; Ribeiro et al. 2015).

7.2 Triatomine–Host Blood

The second site of the vector–host interface relevant during blood feeding is formed between insect anterior midgut contents (intestinal and salivary molecules) and vertebrate blood components. Midgut microvilli are covered with an unusual structure called perimicrovillar membrane in hemipterans, extending toward the luminal compartment with a dead end. The perimicrovillar membrane is well developed in blood-fed, but is poorly developed in unfed insects (Terra et al. 1996).

The blood ingested (plus saliva) during triatomine feeding is stored in the wide anterior part of the midgut, from which water and ions are transported to the hemolymph and Malpighian tubules before excretion via the rectum. Concentrated blood passes slowly into the digestive and absorptive part of the posterior midgut in small amounts (Kollien and Schaub 2000). The blood ingested remains in the anterior midgut until the end of digestion, which may take several days. For example, *T. infestans* adult females can ingest a blood meal ~2.1 times their own weight, the digestion of which takes an average of 14 days (Lehane 2005). It is intestinal

phenomena occurring during the blood meal, that may affect blood pumping by the insect cibarial pump, which will be emphasized here.

Blood clotting was triggered experimentally in the anterior midgut by the administration of exogenous thrombin or knockdown of a specific intestinal anticoagulant named brasiliensin (a thrombin inhibitor) in *T. brasiliensis* fourth instar nymphs (Araujo et al. 2007). Upon gene knockdown by RNAi, the bug's blood meal size (WG) on hamsters was considerably reduced (39–55%). After a dissection of the insects, clots were observed in their anterior midgut, while blood was only compacted in control bugs, as 'clots' were easily disrupted by shaking. The presence of fibrinolytic activity in the triatomine midgut was demonstrated by Hellmann and Hawkins (1964), and this activity could explain why the presence of blood clots in the anterior midgut of blood fed *T. brasiliensis*, which had brasiliensin knocked down by dsRNA injections did not prevent blood transference and digestion in the posterior midgut.

Electromyogram analysis revealed that *T. infestans* and *R. prolixus* had a higher EIR/F in comparison to *T. brasiliensis*. The reduced TIR for *T. brasiliensis* was associated with the poor ability of the species to maintain F during blood ingestion, even when the feeding site is favorable (such as mouse lateral tail vein) (Paim et al. 2011). This higher TIR/F value observed for *T. brasiliensis* when fed on mice previously treated with heparin reinforces the importance of maintaining fluid blood in the anterior midgut for successful blood feeding. When *T. brasiliensis* nymphs were knocked down for brasiliensin and fed from lateral tail vein, they showed difficulty during feeding presenting lower TIR, longer NIP and a blood meal size 2x lower than control nymphs. This difficulty observed for silenced insects could be reverted by treating mice with heparin (Paim et al. 2011).

Besides coagulation, other mechanisms such as platelet aggregation can influence blood viscosity in the insect anterior midgut. As thrombin is also a potent activator of platelet aggregation (Ribeiro and Francischetti 2003), it is possible that the reduction in brasiliensin levels in silenced insects elicited platelet aggregation that could have helped in clot formation in the intestine.

Similar results were obtained for the bedbug *Cimex hemipterus*, which showed improved feeding parameters when fed on pigeons previously treated with a systemic anticoagulant (warfarin). *C. hemipterus* presented a blood meal approximately twice as large in size when fed on treated birds (WG = 3.7 mg) as opposed to untreated pigeons (WG = 1.7 mg) (Araujo et al. 2009b).

On the whole, results obtained for *T. brasiliensis* knocked down for brasiliensin support the idea that blood must remain fluid enough during the ingestion period, as backpressure induced by increased viscosity caused by blood clots prevent the successful pumping of blood into the midgut. Once the blood coagulation cascade has been initiated, viscosity rises rapidly during the clotting process (Puckett et al. 2005). Molecules present in the anterior midgut can also exert a hemagglutination effect on erythrocytes (RBC) of certain vertebrate hosts (Pereira et al. 1981; Gregorio and Ratcliffe 1991; Araujo et al. 2009c; Moreira et al. 2018).

When comparing feeding performance of fifth instar *T. brasiliensis* nymphs fed either on humans or *Thrichomys apereoides* (= *Thrichomys laurentius*, an abundant

species of wild rodent that inhabits the same type of rock burrows as *T. brasiliensis* in Northeast Brazil, see Roque et al. 2005), the insects had more difficulty (<EIR and WG) obtaining a blood meal from wild rodents (Guarneri et al. 2011) (Table 1. Lines: 8-9 and 10-11). Similar results were obtained for third instar nymphs fed on rats or *T. laurentius*, where insects fed on wild rodents showed lower TIR than those fed on murine hosts (Araujo et al. 2009b). The first evidence that hemagglutination can interfere with insect feeding came from the observation that *T. brasiliensis* anterior midgut contents are unable to agglutinate RBC from *T. laurentius* blood compared with rat blood.

Thus, experiments were carried out where *T. brasiliensis* specimens were fed on artificial diets prepared with cattle RBC (such as *T. Laurentius*, RBC are not agglutinated) or rat RBC (which were agglutinated) resuspended in plasma from rat blood, to maintain similar characteristics (e.g., hematocrit, RBC size, plasma viscosity). Higher TIR values showed by insects fed on rat RBC suspension demonstrated that the occurrence of intestinal hemagglutination can positively impact bug feeding performance. The inability of *T. brasiliensis* to agglutinate *T. laurentius* RBC may partly explain this great difficulty (low TIR/EIR and a reduced blood meal) to obtain a blood meal from this wild rodent (Araujo et al. 2009c; Guarneri et al. 2011).

The impact of intestinal hemagglutination on obtaining a successful blood meal, demonstrated for triatomines, is also present in other hematophagous species. Feeding performance in three *Anopheles* species was higher in those with intestinal hemagglutination activity (Chege and Beier 1998). For example, *A. albimanus* and *A. freeborni* agglutinate human RBC feed more rapidly than *A. gambiae,* which is lacking the ability to agglutinate human blood.

The effect of hemagglutination on blood feeding performance may be related to the alteration of blood viscosity. Blood flow decreases dramatically after passing through the narrow anterior region of the food canal and reaching the broad anterior midgut; it even stops in some regions. Under these conditions, the rapid agglutination of RBC together with the deposition of agglutinates formed in the intestinal wall reduce the number of suspended red blood cells ("hematocrit") within the insect anterior midgut. Due to a decreased viscosity of the posterior part of the liquid feed column, these regions with reduced hematocrit create lower resistance for the cibarial pump to propel blood through the food canal.

The natural capacity of erythrocytes of certain mammalian species to form rolls, also known as erythrocyte aggregation (not hemagglutination), can also change blood viscosity at low shear conditions. Erythrocyte aggregation varies greatly among vertebrate species, with the highest level observed in horse blood followed by cat, human, dog, and pig blood. Low levels of erythrocyte aggregation were observed of RBC from rabbit, mouse, rat, cattle, and sheep (Weng et al. 1996; Windberger et al. 2003).

As soon as blood is exposed to the anterior midgut wall, the host complement system is activated. The alternative pathway is triggered by carbohydrates from the glycocalyx of intestinal cells, whereas the classical pathway is triggered by the nonspecific binding of natural antibodies to these carbohydrates or other intestinal mol-

ecules. The presence of carbohydrates covering intestinal membranes can also trigger the lectin pathway by binding to the mannose-binding protein. As insect enterocytes forming the intestinal epithelium are in a single layer, preventing complement activation is essential for the insect to survive, as it could lead to the rupture of the digestive tract and even to insect death. Both saliva and intestinal contents from all triatomines studied to date (*T. brasiliensis*, *T. infestans*, and *R. prolixus*) are capable of inhibiting C3b deposition by classical and alternative pathways of the human complement system (Barros et al. 2009).

8 Final Comments

A major proportion of triatomine total contact time (TCT) is used for pumping blood (IP) from the host skin blood vessel to the insect anterior midgut. Two physical sites of the triatomine–host interface are relevant during this process: a) the triatomine "functional mouth" and host endothelium and b) the insect anterior midgut and host blood. Considering these sites, it is possible to identify features, important in the triatomine–host interaction, that modulate insect feeding performance.

Effective intake rate (EIR) is the feeding parameter that affects the time of contact with the host the most in triatomines. Differences in EIR (F × QLC) between triatomine species can vary greatly if any of the factors such as host species, evolutionary stage, and feeding site are considered in these comparisons. For example, the EIR of fifth instar nymphs fed on mouse abdominal skin varies from 3.5 mg/min for *R. neglectus* to 21.3 mg/min for *T. infestans*, representing a 6.1-fold difference in EIR.

Although QLC (i.e., the bug's intrinsic characteristics) is the main parameter associated with differences in EIR found between triatomine species or among nymphal stages, the impact of host change (pigeon × mouse) on EIR within the same species/stage is associated more with F variation.

These findings show that the main indicator of the impact of external factors, such as host species (avian or mammals), feeding site characteristics (e.g., vessel diameters), and/or blood characteristics in the midgut environment (e.g., coagulated/uncoagulated, aggregated/unaggregated) on triatomine feeding performance is cibarial pump frequency.

Since the food channel is continuous and blood is incompressible any obstacle to blood flow between the triatomine functional mouth and the anterior midgut can interfere with the blood intake rate. Therefore, to produce a true model capable of describing triatomine blood feeding biomechanics, it is necessary to consider the entire insect gut, since current models include the initial parts of the alimentary canal only.

The modification of vector blood feeding performance by parasites to enhance parasitic circulation appears to be a common strategy found in many parasite–vector associations; thus, an additional aspect worth investigating is whether intestinal colonization by pathogens or microbiota affects the ability of the arthropod to feed.

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Blood Digestion in Triatomine Insects

Pedro L. Oliveira and Fernando A. Genta

Abstract Triatomine insects had a fundamental role in the establishment of essential concepts of insect physiology, especially thanks to the use of *Rhodnius prolixus* as a model for basic research in the last century. The major unique feature that made triatomines excellent models is their strictly hematophagous way of life, as molting in nymph stages and oogenesis in the adult are triggered by a blood meal. Therefore, development and reproduction are strictly controlled in an on/off fashion by the arrival of the voluminous blood meal at the digestive channel. However, these insects are less popular in the science arena due to the scarcity of genetic and molecular data. The last few years, however, have manifested a change in the field due to the appearance of large datasets of genomic, transcriptomic, and phylogenetic molecular information, as well as a series of new methodological advances. Here, we tried to make a brief connection between the classical outline of blood digestion physiology in triatomines and this new science scenario, trying to identify critical information that is still lacking.

Keywords Triatomine · Chagas disease · Midgut · Digestion · Digestive enzymes · Hematophagy

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1 Triatomine Evolution—You Are What You Eat but Also What Your Ancestors Used to Eat

Triatomines originated from a lineage of predatory reduviids (Monteiro et al. 2018), presumably driven initially into a vertebrate nest-dwelling habit via the search of invertebrate preys that made their living from a multitude of kinds of body remains, such as fur, skin or dung, or from leftovers from the nest owner food (Schofield 2000). This first approximation has been hypothesized to go through a transition period of facultative hematophagy to the obligate hematophagy that is the distinctive life trait of the triatomines. The putative reduviid ancestors, in turn, were sap-sucking hemipterans that were highly adapted to insert their mouth parts into plant vessels and ingest a very diluted solution of basic monomers (carbohydrates and amino acids), almost devoid of proteins and complex polysaccharides.

Contemporary triatomines are completely committed to blood feeding, as in the absence of a blood meal, no molting occurs in all stages, and no eggs are produced by the adult female. However, several lines of evidence indicate that, in nature, triatomines are not as strictly hematophagous as they were traditionally described to be. Several anecdotal observations in both the laboratory (Noireau et al. 2009; Alves et al. 2011) and field (Salvatella et al. 1994) testified that they are capable of feeding on the hemolymph of diverse arthropods, including other kissing bugs while they were feeding (called cleptohematophagy). Hemolymph feeding increased survival in the absence of a blood meal (Alves et al. 2011). Interestingly, the triatomine saliva has a nonidentified component that is capable of promoting temporary insect paralysis upon injection in the hemocoel of other insects (Alves et al. 2011), suggesting that, more than just a relict habit that was preserved from their predatory ancestors, this strategy is an adaptative life trait that still confers increased fitness to present-day triatomines. In addition, the ingestion of plant tissues and artificial sugar meals by *Rhodnius prolixus* was recently recorded in the laboratory, but the physiological significance of these findings is still unclear (Diaz-Albiter et al. 2016).

2 Triatomine Midgut Morphology: Unique Compartments

Triatomine insects feed several times their size before feeding in a single meal (Friend et al. 1965), and their digestive apparatuses show several morphological and biochemical features that contribute to blood feeding and blood digestion that are regarded as adaptations to this hematophagous way of life. However, the triatomine midgut clearly follows the hemipteran midgut body plan, and this nonhematophagous ancestry was a source of (pre)adaptations, or exaptations, a more precise term suggested by Gould and Vrba (2016), that allowed occupation of this ecological niche but also predefined and limited the evolutionary space solutions that were possible (Terra 1990). Accordingly, the alimentary channel comprises an anterior portion of ectodermic origin, with a chitinous sclerotized surface comprising a pharynx

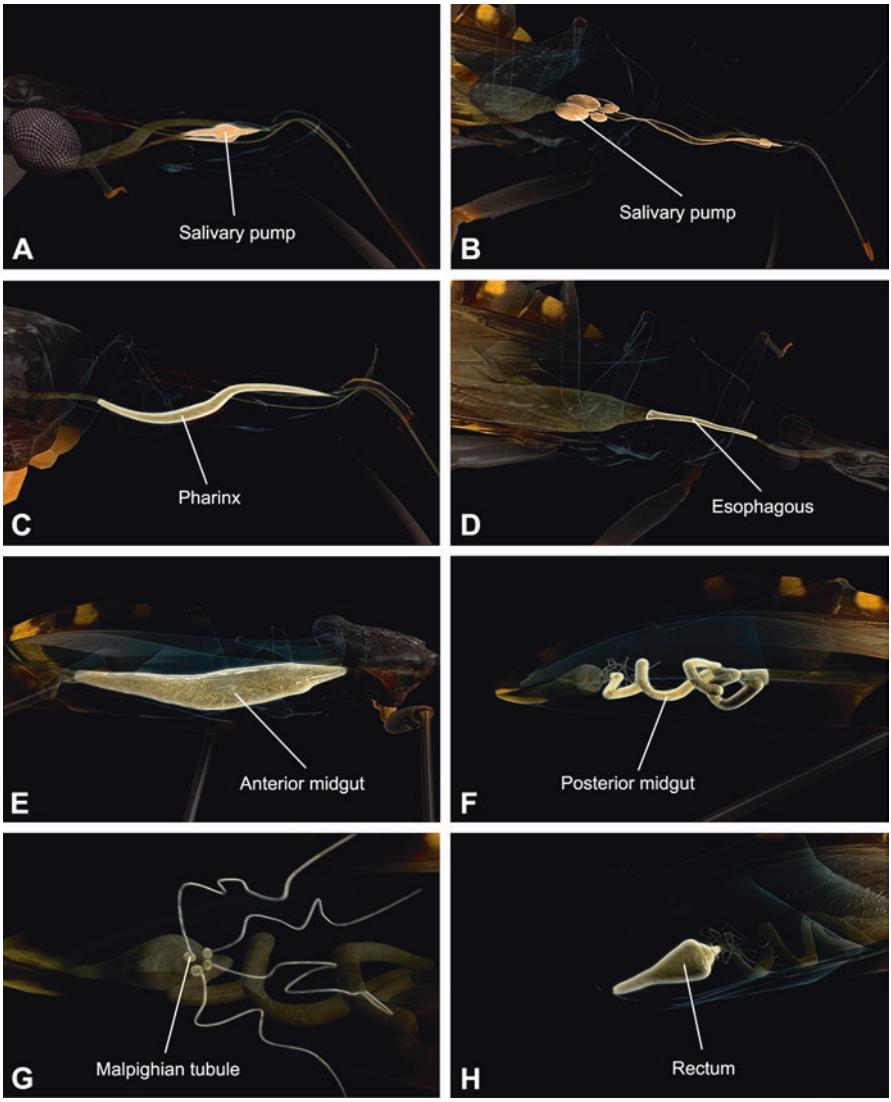

Fig. 1 Illustrative images of the alimentary canal of triatomines and structures associated. In bright, some specific compartments are highlighted, as (**a**) salivary pump, (**b**), salivary glands, (**c**) pharynx, (**d**) esophagus, (**e**) anterior midgut, (**f**) posterior midgut, (**g**) Malpighian tubules, and (**h**) rectum. Images gently provided by Genilton José Vieira (Oswaldo Cruz Institute), extracted from the video "Triatomines: Vector of Disease" (Vieira 2019)

and esophagus, followed by endodermic midgut (which can be further subdivided into a variable number of chambers in different hemipteran groups but typically has two segments in the triatomine subfamily, the anterior and posterior midgut). The end of the digestive apparatus, the hindgut, is again ectodermic, where the

Malpighian tubules discharge water and large amounts of nitrogen waste products, mainly uric acid (Fig. 1).

The initial segments of the digestive apparatus are frequently described just as a channel linking the mouthparts to the midgut. However, these segments play an important role in generating a large negative pressure that is essential to suck blood. Although there is some uncertainty in estimating the precise value of the pressure, it has been estimated to be as high as 14 atm (Lehane 2005). The very high blood flow rates generated by this pressure have a clear adaptative value by reducing the contact time with the vertebrate host and involve the action of powerful muscles in the cibarial pump, which is a very specialized structure found at the initial part of the food channel in the head of the insect. Recently, however, Lahondere et al. (2017) elegantly demonstrated that these segments have a key role in promoting heat loss, which involves a morphological close association with the insect heart, leading to heat transfer to the flowing hemolymph, followed by dissipation through close contact between the heart sinus and the cuticle of the head. It was shown that temperatures in the range of the mammalian body or even slightly above 30 °C are highly deleterious to *R. prolixus* (Okasha 1970; Okasha et al. 1970). Heat loss by this unique mechanism can minimize/prevent thermal stress to the internal organs, especially the anterior midgut, and it was shown by these authors that ablation of the heart compromised heat loss and led to increased expression of heat shock proteins (Lahondere et al. 2017).

The anterior midgut is frequently described as a reservoir chamber where the blood meal is kept undigested at neutral pH and then gradually transferred to the posterior midgut, a long acidic segment where most of the degradation of the blood proteins occurs. However, recently, several lines of evidence have shown that the anterior midgut is the site of a wide range of metabolic activities, including water absorption (Barrett 1982) and secretion of anticoagulant and antimicrobial proteins, but it is also the place of secretion of some digestive activities (Ribeiro and Pereira 1984) and where a large microbiota develops (see below). This section is characterized by an exceptional capacity to increase in size to accommodate the voluminous incoming blood meal, which involves distending this highly folded epithelium plus opening of the spaces of the basal labyrinth and flattening of the columnar cells to increase the organ volume several fold. This structural alteration is complemented by a remarkable elasticity of the muscular network that involves the continuous thick basal lamina that supports the entire midgut epithelium. As professional secretory cells, midgut epithelial cells have a highly developed rough endoplasmic reticulum (ER). Before a blood meal, the ER is clustered in large stacks organized as concentric whorls, which change to a more dispersed pattern after the blood meal, followed by reappearance of the spiral-like ER organization after a few days (Billingsley and Downe 1983).

Midgut epithelial cells of both segments display microvilli with an unusual heptalaminate membrane arrangement at the apical surface, which is connected to a unique conserved hemipteran anatomical feature that is the presence of perimicrovillar membranes (PMVMs) (Billingsley and Downe 1983; Billingsley 1988). PMVMs are lipid membrane bilayers with low protein content that ensheath the

microvilli with a dead end. They are secreted as the Golgi apparatus releases double membrane vesicles that merge with the microvillar membrane. In this respect, the outer membrane of these vesicles fuses with the microvillar membrane, and the inner membrane of these vesicles fuses with the PMVM (Terra and Ferreira 2005; Ferreira et al. 1988a, b). PMVMs are found in both the anterior and posterior segments of the triatomine midgut, although they are more prevalent in the latter, and their formation is greatly increased after blood intake, being continuously delaminated toward the lumen and forming abundant extracellular membrane layers (ECMLs), a dominant process that takes place during all periods in which there is active digestion. These phospholipid membranes fuse in the lumen close to the epithelia and are thought to define a complete separation of the epithelium from luminal contents (Billingsley 1990).

The gut of most insects has a peritrophic membrane or a peritrophic gel, which prevents epithelial cells from directly contacting the food bolus. In most insects the major PM function is to enhance the efficiency of digestion by avoiding the adsorption of undigested food onto the midgut surface (blocking transporters) and by allowing a countercurrent flux of fluid that hampers digestive enzyme excretion (Bolognesi et al. 2008). The peritrophic membrane is an extracellular membrane or gel composed of chitin and proteins, among which special attention has been given to peritrophins, a group of proteins characterized by a CBM14-type (PFAM PF01607) chitin-binding domain (Terra and Ferreira 2005). This structure has also traditionally been ascribed a protective function against mechanical damage by particulate food, but some work has provided evidence that it also acts as a barrier against chemical insult by food components or as a way to limit exposure to the abundant microbiota that are usually found in the gut luminal space. PMVMs may have assumed some of these functions in the gut of hemipterans and are thought to help in the absorption of amino acids (Terra 1988; Terra and Ferreira 1994). Hemipterans lack peritrophic membranes, and it has been proposed that the plant sap-sucking habit of the hemipteran ancestors was accompanied by loss of both peritrophic membranes and digestive serine proteases as an adaptation to a diet composed of diluted monomeric solutes, such as sugars and amino acids, and poor in macromolecular components, especially proteins (Terra and Ferreira 1994).

The posterior midgut has long been recognized as the compartment dedicated to protein degradation (Garcia et al. 1978; Houseman and Downe 1982), which is accomplished by lysosomal-type proteases at an acidic pH (Billingsley 1988). Among blood proteins that are hydrolyzed, hemoglobin is the most abundant, and the cleavage of its polypeptide chain leads to release of the heme prosthetic group that is promptly converted to hemozoin, a crystalline aggregate initially thought to be exclusively found in the plasmodium parasite. The formation of hemozoin is an adaptation to detoxify free heme, which is a pro-oxidant molecule, thus preventing oxidative stress (Oliveira et al. 1999). Most of the heme (>95%) in the midgut is found as hemozoin (Stiebler et al. 2010), whose formation seems to involve crystal nucleation at the PMVMs and extracellular membrane layers that are found in the midgut lumen (Oliveira et al. 2000). The mechanism of hemozoin formation is still an unsolved question for which several putative promoters have been indicated in

plasmodium, and one report has indicated a role of an alfa-glucosidase bound to the perimicrovillar membrane (Mury et al. 2009). However, other reports suggest that less specific mechanisms may involve lipids (Stiebler et al. 2014; Sandlin et al. 2016).

The hindgut, also referred to as rectum or rectal ampoule, is a sac-like segment comprising a stratified non-absorptive epithelium lined with a thin smooth layer of chitin and wax (Schmidt et al. 1998). Its anterior part connects with the posterior midgut and is the site of discharge of the urine copiously produced by the four Malpighian tubules after a blood meal. The large volumes of urine are accommodated due to the remarkable distension capacity of this organ and are eventually actively discharged due to contraction of the muscular coat that surrounds the epithelium. Here, pH is close to neutrality, and the luminal content is depleted of nutrients and may be either a clear, transparent solution or a suspension dominated by crystals of uric acid, hemozoin, or both.

3 Digestive Enzymes and Metabolite Handling

In general, one of the hallmarks of the studies of insect digestion is the connection between spatial compartmentalization of the steps of complex food molecule breakdown with the specificities of enzymes involved in each step (Terra and Ferreira 2005). In this respect, this whole physiological process may be divided into three main phases: initial, intermediate, and final digestion. Initial digestion is the breakdown of complex and large molecular weight macromolecules to intermediate size products, such as oligomers. Intermediate digestion is the reduction of oligomers to dimers, and final digestion is the release of monomers from dimers and their absorption by midgut cells. Compartmentalization of enzyme activities is achieved by differential expression in separate compartments of the gut, restriction of activities to the ectoperitrophic space, or attachment of specific proteins to cellular membranes on microvilli. In triatomines, there is a strong physiological distinction between the anterior and the posterior midgut, with several enzymes being preferentially produced in one of these compartments. In addition, there are some examples of activities that are associated with the perimicrovillar or microvillar membranes, mainly enzymes involved in the final breakdown of sugars and peptides, such as alpha-glucosidase (PMM), alpha-mannosidase (MM), and aminopeptidase, the last of which is trapped in the space between PMM and MM (Ferreira et al. 1988b).

Proteins account for more than 85% of blood dry-weight, and not surprisingly, studies on digestion of the blood meal have concentrated on identification and characterization of peptidases. Peptidases are the main enzymes responsible for the hydrolysis of peptide bonds in proteins and peptides. They are classified depending on the catalytic group/catalysis mechanism, action pattern, and specificity of the substrate. The main groups of insect intestinal peptidases are the serine-, cysteine-, aspartic-, and metallopeptidases (Rawlings et al. 2018). These enzyme activities may also be endopeptidases, cleaving inner portions of the peptide chain, or exopeptidases, recognizing the amino- or carboxy-terminal residues of the substrate.

Additionally, dipeptidases are specific enzymes with activity against dipeptides. The specificity of peptidases relies on the recognition of individual amino acid residues of the substrate by substrate pockets in the active site of the enzyme (Terra and Ferreira 2005; Henriques et al. 2017).

Characterization of enzymatic activity in earlier studies revealed that the enzymes from the triatomine digestive apparatus were enzymes with an acidic optimum pH, similar to lysosomal enzymes, comprising endopeptidases of the cysteinyl peptidase and aspartyl peptidase families and exopeptidases of the amino and carboxy peptidase families (Garcia et al. 1978; Houseman and Downe 1982; Ferreira et al. 1988a). The similarity indicated by enzymatic properties was confirmed and additionally shown to be derived from ancestry, as sequence homology was observed when the first sequences were obtained by the conventional molecular cloning approach (Kollien et al. 2004). Genomic and transcriptomic data that appeared in the last few years suggested that the activity of these enzymes may involve several genes from each family, which were produced by extensive gene duplication. However, from the data available, mainly that from *R. prolixus* (the only triatomine for which there is a genome available today) as well as some transcriptome data available for both other triatomine species (Ribeiro et al. 2014; Mesquita et al. 2015) and nonhematophagous hemipterans, it was shown that the evolutionary path that led to the present day copies was not similar in each enzyme family. The members of the aspartyl peptidase A1 family (18 genes in *R. prolixus*) showed significant gene duplication that resulted in a triatomine-specific branch, raising the possibility that this event may have been a process involved in the evolution of blood digestion during diversification of this subfamily. In contrast, members of the cysteinyl C1 peptidases and metallopeptidases of the M17 leucine aminopeptidase family, although comprising a similar number of genes (17 and 13, respectively), did not cluster in a triatomine clade, suggesting that the gene expansion in these clades occurred before the advent of hematophagy but still in the diversification of the hemipteran lineage (Henriques et al. 2017).

The simplest reason for this trend toward gene family expansion is to allow increased gene expression, which would obviously help to digest a large protein-rich meal. However, apparently different members of the same enzyme family do not seem to have an identical time course of expression, which would suggest non-identical roles (Henriques et al. 2021). Alternatively, multiple enzymes may also provide slight variations in secondary cleavage specificity for these enzymes, allowing optimized hydrolysis for a wide range of protein substrates and eventually for use of different vertebrates as sources of blood. Comparative analysis of the active site of A1 family aspartic proteases in *R. prolixus* reinforces this hypothesis, as there is no conservation of residues putatively involved in substrate binding (Henriques et al. 2017).

Proteins are the main component of vertebrate blood, and digestion of the blood meal is followed by a large flux of free amino acids, which are transported by amino acid transporters, some of which were identified in transcriptomes (Ribeiro et al. 2014). In addition to the canonical role of amino acids as basic building blocks that will sustain *de novo* protein synthesis for growth, molting, or reproduction, some

metabolic aspects related to the ingestion of blood have arisen from that transcriptomic analysis. A large predominance of amino acid degradation pathways compared to those of synthetic pathways appeared in the gut tissues, a result that was confirmed experimentally for the tyrosine/phenylalanine pathway in Sterkel et al. (2016). These findings point to a transamination/deamination network that should funnel amino acid carbon skeletons toward other pathways, such as substrates for the tricarboxylic acid cycle, but also fuels other pathways, such as gluconeogenesis and *de novo* synthesis of fatty acids (Ribeiro et al. 2014). It is noteworthy to mention here that this metabolic profile could compensate for the relatively poor content of carbohydrates in vertebrate blood (only approximately 0.1 %), contrasting with the high demand generated not only by energy-producing metabolism but also by the need for biosynthesis of chitin, a major structural component of insect exoskeleton and eggshell.

Recently, it was shown that pathways for the degradation of aromatic amino acids are highly overexpressed, especially in the anterior midgut of *R. prolixus*. Although amino acids are essential nutrients, overload due to the excessively large amount of protein ingested in a blood meal seems to be a threat to homeostasis, and inhibition of the tyrosine degradation pathway by either RNAi gene silencing or selective chemical inhibition of rate-limiting enzymes was highly deleterious to the insect. However, the toxicity of the inhibitors seems to rely on active digestion of a blood meal and on the very high free tyrosine levels in hemocoel (Sterkel et al. 2016). Interestingly, this essential role of tyrosine degradation seems to be an adaptive trait shared by evolutionary convergence between several phylogenetically distant groups of blood-feeding insects (Sterkel et al. 2017).

Despite the overall predominance of proteins in the chemical composition of blood, lipids are also a relevant substrate, accounting for approximately 2% of blood dry weight. Dietary lipids may be storage (triacylglycerides) or membrane lipids (phospho- or glycolipids), which are respectively digested by triacylglyceride lipases (TG lipase) or by a combination of phosphatases/glycosidases and TG lipases, in the case of phosphor/glycolipids (Terra and Ferreira 2005).

In triatomines, neutral lipids are apparently hydrolyzed to glycerol and free fatty acids by a TG lipase, whose activity increases after a blood meal (Canavoso et al. 2004; Grillo et al. 2007). Gene candidates have been indicated, but experimental identification of which enzymes are actually hydrolyzing these substrates is still missing (Gondim et al. 2018). Together with the increase in lipolytic activity, there is also an increase in the expression of cytosolic acyl-CoA-binding proteins that are involved in activated fatty acid intracellular trafficking (Majerowicz et al. 2016). Interestingly, in contrast to amino acid metabolism, where degradation pathways predominate over biosynthesis, although lipid oxidation enzymes are also expressed in the digestive tissue, several genes involved in biosynthetic pathways are highly expressed, both for triacylglycerol and phospholipid synthesis. Indeed, lipid droplet accumulation was observed in the anterior midgut of *Panstrongylus megistus* after a blood meal, even before the preceding rise in lipase activity (Canavoso et al. 2004). Marked accumulation of triacylglycerol in the digestive tissue has been documented both as lipid accumulation, enzyme activity, and incorporation of radiolabeled fatty

acids and as expression of genes of biosynthetic pathways (Gondim et al. 2018). Active phospholipid biosynthesis has also been observed on the midgut for dietary FAs (Canavoso et al. 2004; Bittencourt-Cunha et al. 2013) and also being fueled by lipids circulating in the hemocoel, which are delivered by the main insect lipoprotein, lipophorin (Atella et al. 1995, 2000). The need for a large lipid supply to the midgut, even mobilizing internal reserves, indicates a metabolic flux that works in a distinct and even opposite direction when compared to other insects where most of the dietary lipids are converted to neutral lipids and exported to the hemolymph and other tissues, specially to the fat body (Turunen 1975; Fernando-Warnakulasuriya et al. 1988; Arrese et al. 2001; Canavoso et al. 2001) and may reflect the specificity of triatomine insects that rely on a voluminous phospholipid membrane system, the PMVM, and the ECML (Bittencourt-Cunha et al. 2013), to isolate the gut cells from the content.

Another relevant aspect of midgut physiology concerning lipid metabolism is the deposition of a wax layer in the hindgut, in line with its ectodermic origin and the presence of a cuticular layer. Specifically, this wax layer of the hindgut has been ascribed a role in the association with *T. cruzi*, which is apparently mediated by hydrophobic interactions (Schmidt et al. 1998). Although the chemical composition of the hindgut cuticle has not been experimentally characterized, its appearance under electron microscopy is indicative of long chain hydrocarbons, a hypothesis that received support from the identification of high expression levels of transcripts coding for elongase in this intestinal segment (Ribeiro et al. 2014).

Despite sugars being a minor blood component, several sugar-digesting enzymes have already been reported in triatomines and characterized to some extent, mainly in *R. prolixus*. Sugar-hydrolyzing activities, such as those of glycosidases or glycoside hydrolases, are classified based on the anomeric configuration of the cleaved glycosidic bond (alpha- or beta-), on the portion of the substrate recognized (endo- or exoactivities), and on the specific saccharide composition of the substrate or residue near the scissile bond (e.g., glucanase, glucosidase, or mannosidase). In this respect, an enormous variety of activities are commonly found in the digestive process of insects (Terra and Ferreira 2005). Glycoside hydrolases are classified based on amino acid similarities in more than 165 families (Lombard et al. 2013).

The initial observation of several glycosidase activities by Ribeiro and Pereira (1984) in fifth-instar nymphs pointed out that the anterior and posterior midgut show strikingly different levels of activities, with most enzymes being more active in the anterior midgut. In addition, for several enzymes, anterior midgut levels are induced in the first 2 weeks after blood digestion. In this category, we found beta-glucosidase, beta-galactosidase, alpha-fucosidase, and beta- and alpha-mannosidases. Other activities showed induction in the anterior midgut only after the third week after blood feeding, including those of alpha-glucosidase, alpha-galactosidase, alpha- and beta-N-acetylgalactosaminidase, and beta-N-acetyl-glucosaminidase (Ribeiro and Pereira 1984; Henriques et al. 2021). It is important to consider that for some activities, the levels were higher in the posterior midgut, such as those of beta-mannosidase, alpha- and beta-N-acetylgalactosaminidase, and beta-N-acetyl-glucosaminidase (Ribeiro and Pereira 1984; Henriques et al. 2021).

Additionally, some activities show very different temporal patterns depending on the tissue considered (beta-glucosidase, beta-galactosidase, alpha-fucosidase, and beta-mannosidase). Nevertheless, the increase in some glycosidase activities in the first two weeks suggests participation of these enzymes in the digestion of dietary components, such as glycoproteins or glycolipids from blood or glycoconjugates derived from microorganisms that are components of the gut microbiota, which increase enormously after feeding (see below). Activities that increase at 3 or 4 weeks after feeding seem to be related to molting, a pattern that is followed by lysozyme production in the anterior midgut. Interestingly, lysozyme activity in the posterior midgut increases in the first week after feeding (Ribeiro and Pereira 1984; Henriques et al. 2021), which can be related to degradation of the bacterial cell walls of gut microbes, especially that of the symbiont *Rhodococcus rhodnii* (see below).

Interestingly, recent evidence documented ingestion of vegetal fluids by *R. prolixus* in the laboratory, leading to positive impacts on the physiology of the insect (Diaz-Albiter et al. 2016). In addition, *R. prolixus* actively ingested artificial sucrose solutions, but the participation of digestive glycosidases in this phenomenon has not yet been investigated. It is noteworthy that the role of several glucosidases in triatomines is poorly understood, with some important observations regarding the interaction with trypanosomatids. For example, a recombinant beta-1,3-glucanase produced by *Rhodococcus rhodnii* has been shown to have very detrimental effects against *T. cruzi* (Jose et al. 2013) However, although digestive beta-1,3-glucanases are widespread among insects (Souza et al. 2019), no beta-1,3-glucanase has been described so far in triatomines. An interesting hypothesis that arises from this finding is that the sugar coating of the surviving parasite cells produces structures that are not recognized by the main hydrolytic activities that are present in the intestinal lumen.

4 Proteins and Molecules Without Enzymatic Activity

While digestive enzymes are always at the center of the stage in the literature on the digestive physiology of most insects, digestion efficiency relies on more than hydrolysis of blood components. A first and necessary step is the lysis of red cells, which is apparently promoted by a peptide produced by the anterior midgut that remains uncharacterized (Azambuja et al. 1983), that helps in rendering hemoglobin accessible to proteases in the posterior midgut. However, hemolytic activity of the intestinal bacterial symbiont *Serratia* has also been reported, and the relative importance of each activity has yet to be determined (Azambuja et al. 2004). In addition to the hemolysis of red cells, the activity of proteolytic enzymes is greatly enhanced by the solubilization of food particles, which is promoted by chewing in several animals that is irrelevant for liquid food (such as vertebrate blood). Following this rationale, it is easy to acknowledge how blood coagulation might be deleterious to blood-feeding insects. The saliva of triatomines is a rich source of anticoagulants (Ribeiro

1995; Ribeiro and Arcà 2009), and some saliva is ingested along with the blood meal. However, salivary anticoagulants apparently are not enough to maintain inhibition of coagulation cascade proteases inside the anterior midgut, a task that is accomplished by Kazal-type serine protease inhibitors synthesized in the anterior midgut of several triatomine species (Friedrich et al. 1993; Campos et al. 2004; Araujo et al. 2007; Meiser et al. 2010; Paim et al. 2011). Infestin, the inhibitor found in *Triatoma infestans*, has seven Kazal domains in a row, targeting multiple distinct serine proteases in the coagulation cascade, including thrombin and Factors XIIa and Xa (Campos et al. 2002). RNAi silencing of brasiliensin, a *Triatoma brasiliensis* inhibitor, revealed a role for this protein in feeding efficiency through control of cibarial pump activity. Apparently, this reduced fluidity of the gut content upon silencing of the Kazal inhibitor generated backpressure that compromised pump contraction (Paim et al. 2011). Surprisingly, the authors mention that no obvious reduction in blood meal digestion was observed, which was putatively attributed to a fibrinolytic activity present in the midgut (Hellmann and Hawkins 1964).

R. prolixus transcriptome analysis, however, revealed that other putative secreted proteins (i.e., proteins having a predicted signal peptide) that do not have enzymatic activity are highly expressed in the gut of triatomines, especially proteins that have binding sites for specific types of small molecules, such as lipocalins, odorant-binding proteins, and chitin-binding proteins. The first two groups are relatively small proteins that have binding sites with affinity for hydrophobic ligands. A differential gene expression study performed in *T. infestans* showed that both lipocalins and odorant-binding proteins have their expression levels modulated by *T. cruzi* infection, suggesting relevant roles in gut physiology/immunity (Buarque et al. 2013). The chitin-binding proteins found in the hemipteran gut belong to both the peritrophin family and to the cuticle chitin-binding protein family. The cuticle protein members are expressed in the hindgut and therefore could be performing their canonical role as components of the endocuticle. In contrast, the finding of peritrophins expressed in the gut of insects that do not bear a peritrophic membrane seems challenging. Interestingly, despite the lack of obvious chitin fibers in the gut in all electron microscopy work that has been performed on triatomine guts, one report has provided evidence for chitin synthesis in the midgut of *R. prolixus*, with silencing of the chitin synthase gene compromising blood digestion (Alvarenga et al. 2016). However, their suggestion of the presence of chitin in the midgut conflicts with the lack of identification of chitin fibers in several TEM analysis published by other authors (Billingsley and Downe 1983; Billingsley 1990).

5 Heme, Iron, and Redox Metabolism in the Triatomine Gut

Another aspect of the biology of the midgut that presents a novel molecular scenario with the advent of genomic data is the study of heme and iron metabolism. Absorption of dietary heme was first intuitively hypothesized by Wigglesworth (1943), when he concluded that hemoglobin degradation produced a

katahemoglobin that was transported from the midgut through the hemolymph and accumulated into the eggs. Indeed, half a century later, it was experimentally revealed that a hemolymphatic heme-binding protein from the insect was able to transport heme from the midgut to other tissues and was taken up by the oocytes by means of receptor-mediated endocytosis (Dansa-Petretski et al. 1995; Oliveira et al. 1995; Machado et al. 1998; Paiva-Silva et al. 2002; Walter-Nuno et al. 2018).

How heme from the posterior midgut lumen that is not converted to hemozoin is taken up into epithelial cells it is not known, but the presence of a transporter is a necessary conclusion. However, to date, the only heme transporter ortholog that has been identified is the FLVCR transporter (from feline leukemia virus cell receptor), which has been proposed to function as a heme exporter (Walter-Nuno et al. 2018). Heme uptake does occur, as oxidative breakdown of heme has been reported, involving a unique complex pathway that involves the addition of two cysteine residues to the porphyrin ring, followed by cleavage by a heme oxygenase (HO) enzyme (Paiva-Silva et al. 2006). This reaction resulted in the release of free iron, which is a potential promoter of the formation of reactive oxygen species (ROS) by means of several reactions, the most famous of which is the Fenton reaction that leads to the production of the very reactive hydroxyl radical. Coherent with this, ferritin silencing resulted in high production of ROS, indicating that iron storage is a necessary counterpart to heme degradation in midgut cells. These findings suggest the need for a close interplay between heme uptake by an elusive transporter, degradation by HO, iron storage by ferritin, and heme export by FLVCR to avoid heme or iron-induced oxidative stress. More than 30 canonical heme and iron eukaryotic genes were identified in the *R. prolixus* genome, and gene silencing resulted in severe phenotypes, highlighting the critical and complex role of heme and iron in the cell biology of hematophagy (Walter-Nuno et al. 2018). A recent comprehensive review on this point has been published that provided a more extensive analysis of the literature on this field, not only covering data from triatomine insects but also providing data on the fate of dietary heme and iron from other blood-sucking insects and noting several aspects that need further investigation (Whiten et al. 2017).

As noted above, iron and heme are potential promoters of oxidative stress; therefore, iron and heme metabolism are closely related to the regulation of redox metabolism in the gut of hematophagous insects. Catalase and superoxide dismutase display relatively high activities in the *R. prolixu*s midgut compared to those in other tissues (Paes et al. 2001). However, ROS levels were diminished in the midgut immediately after the blood meal by reduced ROS formation by midgut mitochondria as part of the signaling pathway triggered by amino acid sensing by the TORC complex (Gandara et al. 2016). This inhibition of ROS production by gut cells was proposed to function as a mechanism that compensates for the nutritional pro-oxidant challenge due to heme and iron intake, but no experimental testing of this hypothesis was provided.

The understanding of the effects of changes in the redox equilibrium on the digestive tissues after a blood meal is further complicated by the production of NO (nitric oxide) by nitric oxide synthase (NOS). NOS expression is induced by the blood meal only in the rectum, but nitrite accumulation is not observed, suggesting

that the enzyme is not activated (Whitten et al. 2007). In contrast, in the other segments of the digestive channel, NO gene expression is not stimulated, but increased nitrite accumulation (from NO production by NOS) is observed after a blood meal, indicating activation of the NOS enzymatic activity. The midgut is the major interface with the microbial world, and an interesting possibility is that this blood meal-induced NO formation may be relevant to control microbiota growth as well as pathogen transmission. In line with this possibility, production of NO is modulated by trypanosome infection in gut tissues, although distinct profiles have been reported (Whitten et al. 2007; Castro et al. 2012), showing that the microbial population in the midgut lumen was effectively recognized by the midgut cells.

6 Triatomine Midgut Immunity and Physiology in a Microbial World: Simplicity Turns into a Complex Scenario

The gut of triatomine insects is populated by an abundant bacterial microbiota, and antibacterial proteins are secreted after a blood meal (Vieira et al. 2014). Microorganisms (bacteria or trypanosomes) that inhabit the gut lumen are sensed by the insect immune system by increasing the expression of antimicrobial proteins that are present in the gut epithelia, including gut-specific lysozymes (Ursic-Bedoya et al. 2008), prolixicin, and defensins (Araujo et al. 2006; Vieira et al. 2016). Additionally, it is clear that the immune signaling pathways in the gut are not only activated in response to a bacterial challenge but also can control the bacterial population in the gut, as demonstrated by increased bacterial levels after RNAi gene silencing of immune genes (Mesquita et al. 2015) or administration of an IκB kinase inhibitor (Vieira et al. 2018). Key enzymes in the insect innate immune response are the phenol oxidases, enzymes that catalyze the hydroxylation of phenolic substrates, ultimately leading to the formation of melanin and eventually promoting the killing of pathogens. Not surprisingly, most prophenol oxidases are found in the *R. prolixus* midgut, specifically in the anterior segment (Genta et al. 2010), where most of the intestinal microbiota are concentrated (Eichler and Schaub 2002). Several excellent reviews, including a chapter in this book, have addressed in detail the interaction of the immune system and its antimicrobial protein effectors with both the parasite and the indigenous microbiota (Chapter "The Immune System of Triatomines"); therefore, we will not expand further on this topic.

Since the original description of a symbiotic bacterium in the gut of *R. prolixus* by Duncan (1926) and Wigglesworth (1936), an essential contribution of the microbiota to the physiology of triatomines was acknowledged in the literature and invariably attributed to a single microbial species. This bacterium, initially named *Actinomyces rhodnii*, was renamed *Nocardia rhodnii* and, more recently, based on molecular data, reclassified as *Rhodococcus rhodnii* (Zakrzewska-Czerwinska et al. 1988). The role of the bacteria as a provider of essential vitamins has been an early

hypothesis put forward by Wigglesworth (1929, 1936), but further work provided conflicting evidence on this proposition (Harington 1960; Lake and Friend 1968; Hill et al. 1976). Contrasting with these earlier reports, a *Serratia marcescens* strain was identified as a major component of the *R. prolixus* midgut (Azambuja et al. 2004). The composition of the triatomine microbiota was initially studied using culture-independent methods by the work of da Mota et al. (2012), who confirmed the presence of *Serratia* but found a complex microbial composition not only in *R. prolixus* but also in other triatomine species. Later, this finding was confirmed and extended by other studies for both laboratory colonies and field-collected insects under certain conditions, including a large predominance of specific mutualist symbionts of species from several genera.

The association of triatomines and diverse gut-dwelling microorganisms may have unexplored significance in the adaptation of these insects to the hematophagic habit, with unexpected impacts on the insect genome. Recently, horizontal transference of at least three peptidase genes from microorganisms to the insect genome was confirmed, and these three genes are actually expressed in the midgut (Henriques et al. 2017). Interestingly, one of them is a murein endopeptidase (peptidase family M74), which has a putative role in the degradation of bacterial cell walls and is a candidate to function in the digestion of symbionts or control of bacterial populations in the midgut. However, the physiological role of these proteins has not been clarified in detail.

A comparison of the recent literature with the classical descriptions shows that although microbiota bearing a single bacterial type may be observed (although this finding has not been tested by a culture-independent approach), the more common situation is a consortium of multiple species of bacteria, as found in most other insects. It is interesting to note that the mutualistic interaction of this now diverse microbiota with its invertebrate host, the most striking biological feature of the classical literature on the area, has not been revisited in light of these new findings. An additional layer of complexity has emerged in a metabolomic analysis of the midguts of three species of triatomines, where hundreds of compounds were identified, several of which are likely to be products of bacterial secondary metabolism (Antunes et al. 2013).

7 Final Remarks

The understanding of the physiology of the digestive process in triatomines has greatly benefited from the general technological advances in the field of entomology, especially from the application of genomic, proteomic, metagenomic, and metabolomic tools. The adaptation of protocols in molecular and systems biology for the characterization and manipulation of gene expression and genetic modification of triatomines and microorganisms of their microbiota has opened a new era on the study of the biology of triatomines. This advance allowed experimentation on new hypotheses aiming to unravel the evolutionary adaptations that were decisive in

the origin and success of the blood-feeding habit in these insects and to discover key aspects in the interaction with the parasite *T. cruzi*. The work of several researchers focused on the physiology and biochemistry of triatomines and related groups has revealed that several kissing bug features that are important for hematophagy were in fact preadaptations that are a heritage from their close ancestors, and in this respect, the overall triatomine digestive physiology reflects the evolutionary history of the group. As such, the triatomine digestive system shows many important characteristics that are general trends in the physiology of the insect gut, such as compartmentalization of function and a close relationship with the gut microbiota. Nevertheless, several properties of the kissing bug gut metabolism may turn out to be adaptative convergences shared with other blood-feeding vectors, such as mosquitoes. The current state of the studies of triatomine digestion has opened new avenues for a deeper knowledge of blood digestion and interaction with microorganisms, both from the molecular and systems biology perspectives. This progress will allow the development of new tools for basic studies in vector physiology, ultimately resulting in new strategies for the control of this important group of vectors and the transmission of Chagas disease.

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The Physiology of Sperm Transfer and Egg Production in Vectors of Chagas Disease with Particular Reference to *Rhodnius prolixus*

R. Gary Chiang and Jennifer A. Chiang

Abstract *Rhodnius prolixus* has served since the early part of the last century as an ideal model to explore the physiology of blood sucking insects. Further, comparative studies indicate that the physiological processes described in this insect apply to Triatominae in general. This chapter focuses on the physiology and anatomy associated with two biological processes that occur in adults, sperm transfer during copulation and the endocrine control of egg production following feeding. With respect to sperm transfer, it is now known based on observations reported here that *R. prolixus* does not possess a spermatophore sac that everts into the vagina. Instead, the aedeagus delivers free sperm directly to the base of the common oviduct, and the sperm start their migration to the spermathecae before copulation ends. This information will help to clarify the role of male secretions during copulation. With respect to egg production, it has been observed that (1) the experimental manipulations involving the corporis cardiaca (CC) and the corpus allatum (CA) also affect the major neurohaemal site in the cephalic aorta next to these structures, (2) there is a significant sensory component for egg production provided by the abdominal pressure receptors which are able to continually monitor crop size and (3) circulation through the cephalic aorta is required for egg production after feeding. These factors give rise to a working hypothesis that integrates the endocrine, nervous and circulatory systems at the level of the cephalic aorta – a possible vascular portal system in the female insect.

Keywords Egg production · Corpus allatum · Vagina · Spermatophore · Aedeagus · Rhodtestolin

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1 Introduction

The success of any species is ultimately dependent on their reproductive capabilities, and insect vectors of Chagas disease are no exception. Understanding what ensures a successful insemination, and subsequent egg production, is vitally important for fully appreciating how these insects are able to successfully populate human domiciles and become a major threat to human health. Understanding the physiological basis of Triatominae reproduction forms the underpinnings of research into population control and the spread of disease. This chapter summarizes the present knowledge of sperm transfer and egg production in insect vectors of Chagas disease concentrating primarily on *Rhodnius prolixus*. By documenting what is currently known as a biological fact and what remains a conjecture will help to focus future research on the significant issues in this important area of insect physiology.

There are numerous insect vectors of Chagas disease besides *R. prolixus,* and depending on the location, many are far more important epidemiologically with the most troublesome belonging to the genus *Triatoma* (Galvão 2014; WHO 2017). Yet our knowledge of the physiology of these vectors comes from studies using primarily *R. prolixus* as an insect model. P.A. Buxton (1892–1955) was the first to realize that *R. prolixus* would serve as an ideal model to investigate the physiology of blood sucking insects (Buxton 1930), and it was fortuitous that he appointed V.B. Wigglesworth (1899–1994) as a lecturer in medical entomology at the London School of Hygiene and Tropical Medicine with the mandate to develop insect physiology as a scientific base for the control of harmful insects (Locke 1996). Wigglesworth is considered a founder of insect physiology and was knighted in 1964 for his significant contributions to insect physiology which included a considerable amount of work on *R. prolixus* (Locke 1996). This species became the insect model of choice for many research and teaching laboratories. By the mid-1970s, laboratories around the world had colonies of *R. prolixus*, and many of these colonies were descendants of the colony that Buxton started with insects he obtained from the French entomologist, E. Brumpt (Buxton 1930).

To ensure that the information we have gained by using *R. prolixus* as a model insect in our laboratory can be applied to Triatominae, we explored the structure of the reproductive system in a number of different vectors of Chagas disease (Chiang et al. 2012). In the eight species we examined, reproductive structures were relatively conserved with only some minor variations in gross morphology. Much of this work was verified by Nascimento et al. (2017). We are confident that our findings in the insects from our colony of *R. prolixus*, which have been reared for almost 100 years under laboratory conditions, are applicable not only to *Rhodnius* populations found in the wild, but also to all Triatominae.

2 Sperm Transfer

2.1 Copulation

The mating ritual leading up to the male mounting the female does not occupy much time. Lima et al. (1986) noted that in *Panstrongylus megistus* there appears to be little patterned interaction between mating pairs before copulation begins, and what is considered patterned behaviour involves a sequence of subtle steps carried out by the males (Manrique and Lazzari 1994; Pires et al. 2004; Vitta and Lorenzo 2009). Chemical attractants are common for these insects (Manrique and Lorenzo 2012), and these chemical factors may reduce the need to rely on a patterned approach.

To initiate copulation after coming into contact with a receptive female, the male mounts the dorsal-lateral side of the female facing the same direction. The male holds onto the female by placing his legs on her body while the female stays on the substrate (Fig. 1). With his genital capsule next to the female genitalia, the male lifts his parameres from their grooves at the posterior dorsal rim of his genital capsule

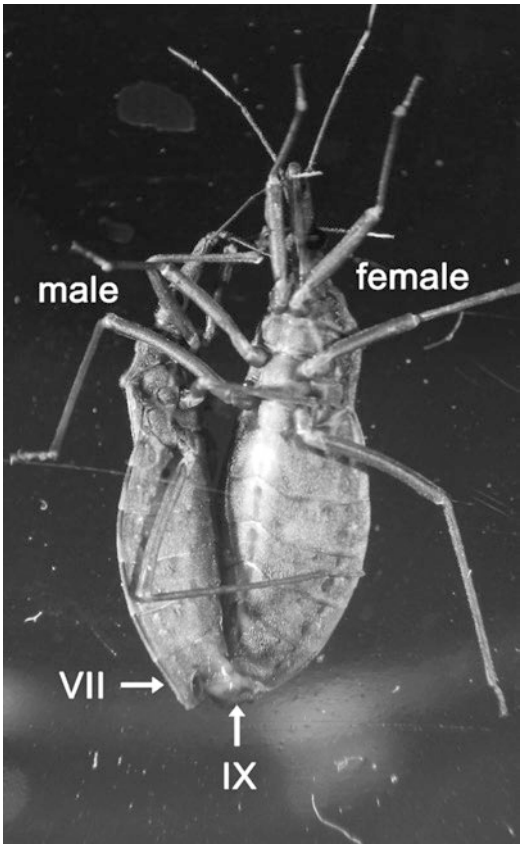

Fig. 1 Copulating *R. prolixus*. The male mounts the female along her side holding her in position by placing his legs on her body. Her legs are free to allow her to hold to or move on the substrate. The male genital segments orient towards the female genitalia by extending out from segment VII. The aedeagus protrudes from the genital capsule (IX) and is inserted into the female. Insects are approximately 18 mm in length

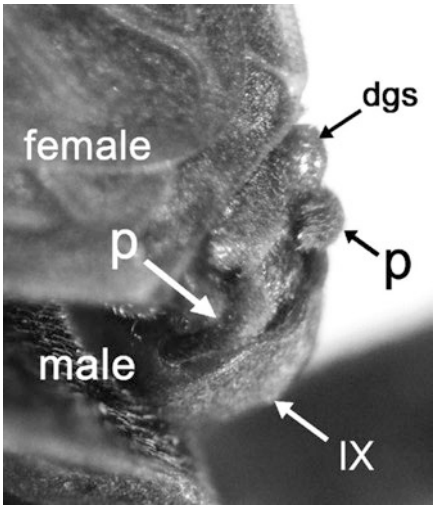

Fig. 2 View of the male and female genitalia and the position of the parameres (p) during copulation. The two parameres extend from the dorsal lateral edge of the genital capsule (segment IX) and hook on to the lateral edges of the dorsal genital segment of the female (dgs)

and extends them towards the female's external genital plates. These parameres then hook onto the dorsal edge of the female dorsal genital plate, and may serve to clasp this segment, and direct the aedeagus into the female (Fig. 2).

2.2 Mechanisms Facilitating Copulation

In many insects, a physical aid in copulation is the paramere which serves as a clasper (e.g. see Moreno-Garcia and Cordero 2008; Myers et al. 2016). For the seed bug, *Leptoglossus occidentalis*, in which the male organ must make its way deep into the female to deliver free sperm directly into the spermathecae, lengthy copulation times of 8 h or more are common (Chiang 2010). In this species, the male claspers are robust and mating pairs remain coupled even after being disturbed. In *R. prolixus*, mating pairs will readily uncouple if disturbed (personal observations) suggesting that the delicate parameres are not associated with a strong clasping mechanism. Such a mechanism may not be needed since the sperm are delivered a short distance to the vagina, and from there they make their own way to the spermathecae after the male withdraws. Nevertheless, even without a well-developed genital clasping mechanism, the male is still capable of staying attached for about 50 min (52 ± 14 min, $n = 26$, Chiang et al. 2013).

The male's ability to maintain copulation for this period of time may be enhanced through a sensory mechanism. This mechanism may involve tactile stimulation of the female's sensory hairs on her ventral abdominal cuticle. Chiang et al. (1992) discovered that gentle stroking of the sensory hairs at this location inhibits the heartbeat, whereas tactile stimulation of other parts of the abdomen does not elicit this response. It is this area of the cuticle which makes contact during copulation. The

inhibition of the heartbeat could be related to a general thigmotactic response in which the insect becomes less responsive to external stimuli when it wedges itself into a confined space, such as a crevice (Wigglesworth 1974). If so, this tactile inhibition may be a mechanism employed by the male to maintain his partner in a receptive state.

The ability to maintain copulation may also be enhanced through a chemical mechanism. A cardio-inhibitor, rhodtestolin, was first isolated from the testes in *R. prolixus* (Martens and Chiang 2010) and later found to be in the male secretions delivered to the vagina (Chiang et al. 2013). This substance is delivered to the female during copulation, and may enhance the general thigmotactic response to calm her. On the other hand, rhodtestolin could be part of the male secretions needed to maintain the viability of the sperm (Khalifa 1950) or to influence the social interactions of the female as has been found in other insects (Avila et al. 2011; Sirot et al. 2009). In addition, because rhodtestolin relaxes the heart muscle, it may also relax the vagina muscles as secretions start to stretch the vagina (see Fig. 11 in Chiang and Chiang 2017b). Tension recordings from the vagina muscles show that they will contract in response to stretch (Chiang and O'Donnell 2009). The male secretions are not ejected immediately after copulation, but remain in the vagina for several hours (Davey 1958). This delay may result from rhodtestolin having a calming effect on the stretched vagina muscles.

3 Sperm Delivered to the Spermathecae

Copulation is successful when the male leaves viable sperm in the spermathecae. In *R. prolixus*, the spermathecae are slender blind-ended tubes that are attached laterally to the common oviduct. Sperm delivered into the vagina must travel up a short distance into the common oviduct, and at the level of the spermathecae, the sperm are directed 90° to enter and fill the slender tubes of the spermathecae, rather than continuing on into the lateral oviducts. How this movement happens in *R. prolixus* is likely by the same process as in other Triatominae since the spermathecae are all described as tubes arising off the common oviduct. There is some variation in the anatomy at the distal ends of these tubes (Chiang et al. 2012; Nascimento et al. 2017), but this variation is minor.

An early study suggested how the spermatozoa might migrate from the vagina and into the spermathecae in *R. prolixus* (Davey 1958). After the spermatozoa are deposited at the vestibulum (the muscular base of the common oviduct which extends into the vagina), the spermatozoa are described as being gulped by bite-like motions of the vestibulum. As they enter into the lumen of the common oviduct, contractions of the common oviduct coinciding with twitches of the spermathecae direct the sperm into the spermathecae. This process assumes the sperm require assistance to migrate and to fill the spermathecae, and this assumption is supported by the observation that the opaque accessory gland secretions of the male enhance the contractions of the female reproductive tract (Davey 1958). Although this

mechanism is intuitively attractive, it is re-evaluated below in light of what is currently known about the structure of the aedeagus and the formation of the spermatophore.

4 The Aedeagus

The aedeagus is the intromittent organ of the male insect and is sometimes referred to as the phallus. In Triatominae, the aedeagus rests inside the genital capsule (Fig. 3), and in lateral view, it has a half-disk shape with the curved side of the disk running along its back. An overview of the aedeagus resting in a pouch in the genital capsule is provided in Fig. 4. Parts of this structure are described below and include the struts, the median plate, the basiphallus and the folds of cuticle inside the aedeagus.

The struts are two bilaterally symmetrical longitudinal strips of sclerotic plates, a feature which is common in many Triatominae (Lent and Wygodzinsky 1979). The struts bend slightly as they follow the curve of the back of the aedeagus. When the aedeagus is in the resting state, the struts terminate close to the distal end of the aedeagus (Fig. 5). At their proximal ends, the struts make a prominent bend towards the body of the aedeagus.

The front side of the resting aedeagus appears as the straight side of the half-disk. The front side is covered mostly by a sclerotic plate (Fig. 5) labelled the dorsal phallotheca plate in Triatominae (Lent and Wygodzinsky 1979). In keeping with its location along the midline of the aedeagus, it has also been referred to as the medial

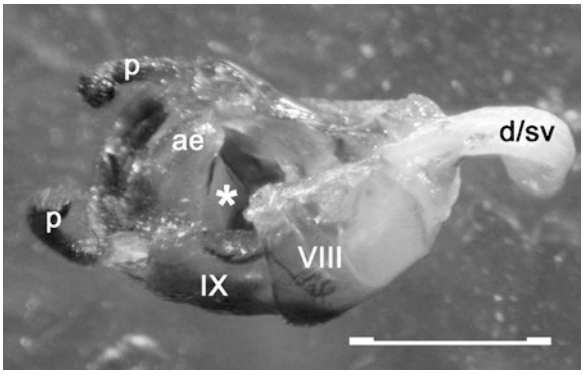

Fig. 3 Dorsal lateral view of the male genitalia with the aedeagus (ae) exposed after removing the soft cuticle and its attached anus and rectum that covers the pouch in which the aedeagus sits in the genital capsule (segment IX). The distal end of the aedeagus is further anterior and lower in the genital capsule when covered by the anus. The asterisk indicates the medial plate covering the straight side of the aedeagus. *p*, parameres extending out from their grooves in the genital capsule; *d/sv*, ducts from the accessory glands and the seminal vesicle; *VIII*, segment VIII, which is the anterior segment of the two segments that make up the male genitalia. Scale bar: 2 mm

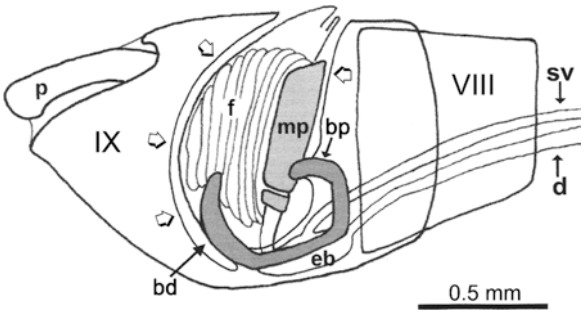

Fig. 4 Line diagram illustrating the position of the resting aedeagus in a pouch (open arrows) in the genital capsule (IX) of the male of *R. prolixus*. The aedeagus is attached to the genital capsule by the u-shaped basiphallus which has two bilaterally symmetrical branches that extend distally (bd) to attach to the base of the aedeagus, and two bilaterally symmetrical branches that extend proximally (bp) to attach to muscles in the genital capsule. *d*, common duct of accessory reproductive glands; *eb*, ejaculatory bulb; *f*, folds of the valve/pump in the aedeagus; *mp*, medial plate covering straight side of the aedeagus; *p*, paramere; *sv*, duct from the seminal vesicle. (Modified from Fig. 3a in Chiang and Chiang 2017a)

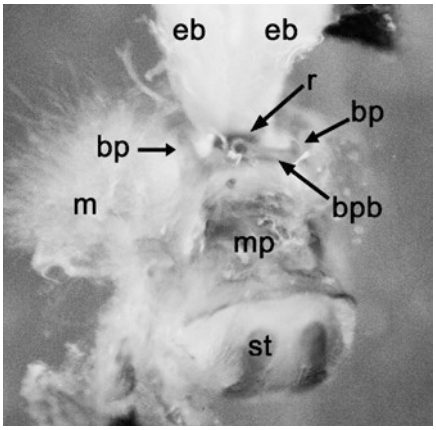

Fig. 5 Light microscope photograph showing the aedeagus with its curved side at the bottom of the photograph, and its distal end pointed towards the viewer. The two proximal branches (bp) of the basiphallus curve into the genital capsule, which has been removed. One proximal branch (on left in picture) can be seen to extend to a mass of muscle fibres (m) which attaches it to the genital capsule, whereas the other branch has been cut to expose the medial plate (mp) of the aedeagus. The ejaculatory bulbs (eb) merge before they attach to the ring to cuticle (r) in the basiphallus which, in turn, is attached by the median bridge (not visible at this angle). The median bridge is attached to the middle of the basal plate bridge (bpb) which connects the two proximal branches of the basiphallus. *st*, distal end of one of the struts of the basiphallus. The width of the aedeagus is approximately 0.5 mm

plate in *R. prolixus* (Chiang and Chiang 2017a, b). The medial plate is attached to sheets of soft cuticle which are arranged into folds along the sides of the medial plate. These sheets of cuticle allow the aedeagus to expand. The medial plate is not completely flat, being slightly convex transversely.

The medial plate is also associated with a pair of thick, bilaterally symmetrical sclerotic arms that are dark brown-to-black in colour. These arms join the proximal side of the inside layer of the medial plate approximately half way between the midline and the lateral edge of the plate. These branches extend a short distance proximally, then turn into the aedeagus. These medial plate proximal branches and their attachments are shown as dotted lines in the phallus of *R. prolixus* by Lent and Wygodzinsky (1979), who also depict similar structures at the same location in other Triatominae.

The half-disk-shaped aedeagus sits on a base which attaches it to the genital capsule (Fig. 6). This base is called the basiphallus and consists of a centrally located rectangular piece of sclerotic cuticle which has attached to it, on both its distal and proximal sides, a pair of branches. Each pair of branches is bilaterally symmetrical. The distal pair extends from the rectangular plate distally and curves around the left and right sides of the base of the aedeagus (Fig. 6). The proximal branches extend proximally becoming progressively further apart until they enter the genital capsule. Here they bend away from each other and curve ventrally then posteriorly before terminating near the sides of the genital capsule. The ends of these branches are attached to muscle fibres (m) located in the genital capsule (Fig. 5). When viewed laterally, the branches of the basiphallus give it an overall U-shape, as depicted in the diagram in Fig. 4.

Figure 5 illustrates how the proximal branches of the basiphallus (bp) are also linked together close to the rectangular plate by a small branch of sclerotic cuticle

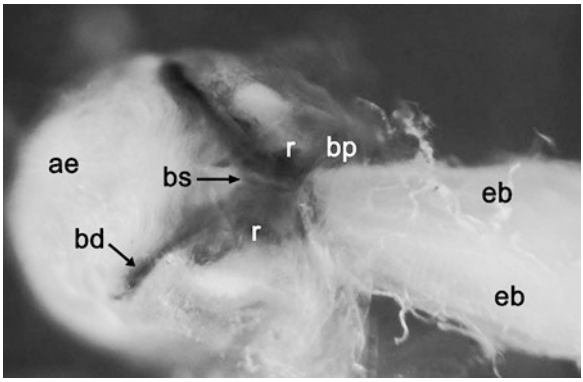

Fig. 6 A light microscope photograph showing the base of the aedeagus (ae) attached to the basiphallus. The two distal branches of the basiphallus (one labelled as bd) extend from the centrally located rectangular plate of the basiphallus (bs). One of the two proximal branches (bp) is seen to extend proximally into the genital capsule, which has been removed. The ejaculatory bulbs (eb) merge to attach to the ring of cuticle in the basiphallus. In this view, the ring of the basiphallus is covered mostly by the rectangular plate, but its sides are visible (r)

which Lent and Wygodzinsky (1979) label the basal plate bridge (bpb). Connected to the middle of the basal plate bridge is the median bridge which extends posteriorly. Under the centre of the rectangle, the medial bridge attaches to a ring of cuticle (Figs. 5 and 6). The two ejaculatory bulbs merge and attach to the proximal side of this ring while a short copulatory duct connects to the ring on its distal side. The copulatory duct carries secretions to the aedeagus and deposits them into a lumen surrounded by a white cuticle that has a complex series of folds. As will be noted below, these folds form a lumen through which the secretions can pass. They also stretch longitudinally to allow the aedeagus to extend.

When the aedeagus does extend out of the genital capsule, it remains firmly attached to the body of the male by the basiphallus. Therefore, the basiphallus is also called the articulating apparatus (Lent and Wygodzinsky 1979). But its function may be more than articulation. Firstly, during copulation, the lumen carrying the male secretions through the ring under the central rectangle turns 135–145° to bend towards the vagina (Fig. 7). The ring in the basiphallus keeps the lumen open preventing it from collapsing as it bends. Secondly, the proximal branches of the basiphallus are attached to musculature in the genital capsule. When the muscles in the genital capsule contract, the tension they generate could be transferred by the basiphallus to the base of the aedeagus.

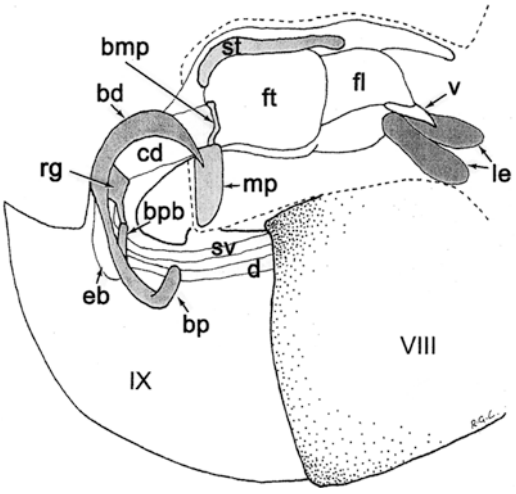

Fig. 7 Line diagram showing the overall appearance of the aedeagus as it extends into the vagina. The wall of the vagina is indicated with the dashed line. *bd*, distal branch of the basiphallus; *bp*, proximal branch of the basiphallus; *bmp*, branch of the medial plate; *bpb*, basal plate bridge; *cd*, copulatory duct; *d*, common duct of the accessory glands; *eb*, ejaculatory bulb; *fl*, region of folded cuticle that can expand longitudinally; *ft*, region of folded cuticle that can expand transversely; *le*, lateral endosoma processes; *mp*, medial plate; *rg*, the cuticle ring in the basiphallus which is attached to the basal plate bridge by the median bridge; *st*, strut of the aedeagus; *sv*, duct of the seminal vesicle; *v*, virga which is attached to the gonopore (not shown); *VIII and IX*, segments of the genitalia

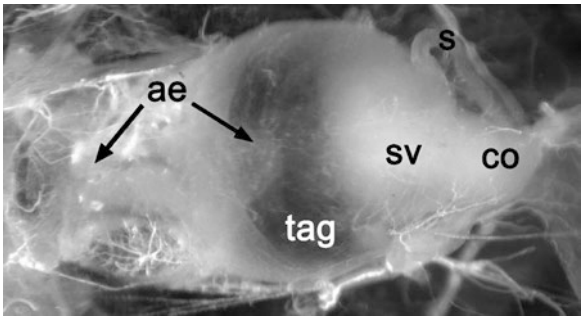

Fig. 8 A light microscope photograph of a dorsal view of an intact vagina as it is being filled with male secretions from the aedeagus (ae). The posterior of the vagina is to the left, and the length of the aedeagus is indicated with arrows. The aedeagus is extended approximately twice its resting length. The light around the aedeagus is refracted by a thin layer of air between the aedeagus and the vagina. The first secretions delivered are from the seminal vesicle (sv) followed by secretions mainly from the transparent accessory glands (tag). At this point during copulation, the seminal vesicle secretions appear to be visible in the common oviduct (co). *s*, one of the two spermathecae attached to the common oviduct

In Triatominae, the aedeagus is divided into a proximal region, the phallosoma, and a distal region, the endosoma (see Lent and Wygodzinsky 1979). The endosoma nests in the phallosoma when the aedeagus is resting in the genital capsule. As depicted diagrammatically in Fig. 7, and photographically in Fig. 8, when the aedeagus is fully extended into the vagina, it lengthens almost twice its size. It also reveals a number of structures that are hidden in the resting state. These include the bilaterally symmetrical folds in the sheets of cuticle associated with the medial plate, as well as a pair of bilaterally symmetrical sclerotic plates at the distal end. Also exposed in the extended state is the gonopore through which the male secretions exit the aedeagus and a v-shaped piece of sclerotic cuticle associated with the most distal end of the gonopore.

The sheets of cuticle associated with the medial plate unfold laterally from the medial plate when the aedeagus extends into the vagina. These sheets have a wing-like appearance, and as they expand, they form a physical barrier between the base of the aedeagus and the inside of the vagina. The medial plate turns towards the bottom of the vagina. In this configuration, the sheets extending from the medial plate together with the medial plate form a collar-like structure around an orifice. The copulatory duct attaches to the proximal edge of this collar and can deliver secretions directly to the aedeagus (Fig. 7).

The distal bilaterally symmetrical plates are elongated, thin and broader at their distal ends also giving them a wing-like appearance. They extend from the lower lateral sides of the aedeagus closest to the floor of the vagina, and have also been referred to as the lateral endosoma processes (Lent and Wygodzinsky 1979). When the aedeagus is fully extended, they are free to move at their base and can reach the vestibulum of the common oviduct or fold backwards. These distal wing-like lateral endosoma processes provide a physical barrier at the distal end of the aedeagus

between the male secretions being deposited in the vagina and the aedeagus. Since the wing-like flaps at the base of the aedeagus also form a seal between the aedeagus and the vagina, the aedeagus does not come into direct contact with the male secretions. During copulation, there is a thin layer of air between the aedeagus and the vagina (Fig. 8). This air escapes when the vagina wall around the aedeagus is punctured.

The gonopore is clearly seen at the distal end of the aedeagus when the aedeagus is extended and secretions are being delivered to the vagina. When the aedeagus is not extended, the gonopore sits at the base of the aedeagus against the inside of the cuticle and appears as a slit with the v-shaped sclerotic plate located at the distal end of the slit. This v-shaped sclerotic plate was labelled the virga in *R. prolixus* (Davey 1958) and a vesica in *Triatoma rubrofasciata* (Lent and Wygodzinsky 1979). Both the slit of the gonopore and the virga are visible through the partially transparent cuticle of the aedeagus. When the aedeagus lengthens, the portion of the cuticle which forms the gonopore slides distally to open at the extreme distal end of the aedeagus. At this point, the virga protrudes from the endosoma like a tongue hanging out of a mouth (Fig. 7).

The virga with the gonopore can slide to the end of the endosoma because they are attached to a tube formed by the highly elastic folds of white cuticle which stretch longitudinally. These folds are continuous with another set of folds which fill up the bulk of the phallosoma. The folds in the phallosoma create a chamber which receives secretions from the short copulatory duct. These folds can easily expand transversely. When the distal end of aedeagus is pulled distally a short distance, it pulls on the tube which expands longitudinally, and this tube rises out of the middle of the folds that expand transversely (Fig. 9). After the aedeagus is released from being pulled, the longitudinal folds snap back to nest in the middle of the transverse folds. If the aedeagus is stretched beyond its normal point, the folds of cuticle are ripped indicating that the folds are not intended to stretch beyond the aedeagus and into the vagina.

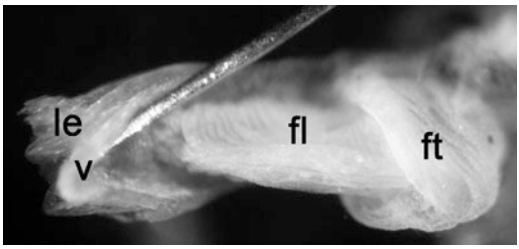

Fig. 9 Light microscope photograph illustrating the folds of cuticle inside the aedeagus revealed by stretching the distal end of the aedeagus with a metal probe. The folds form a tube through which secretions pass through the aedeagus. *fl*, cuticle folds that can expand longitudinally. In this view, the distal ends of these folds attach to the inside of the cuticle that forms the gonopore; *ft*, cuticle folds that can expand transversely; *le*, lateral endosoma process; *v*, virga

5 Sperm Delivery to the Vagina

The delivery of the sperm to the vagina was first described for *R. prolixus* by Khalifa (1950). Assuming the male formed a spermatophore, Khalifa described this apparent spermatophore, removed from chemically fixed vaginas, as a pear-shaped jelly-like mass with a longitudinal slit of spermatozoa at its anterior end where it contacts the base of the common oviduct. This description was supported by Davey (1959), who suggested that the rhythmic contractions of the musculature of the vestibulum at the base of the common oviduct created the longitudinal slit in the spermatophore to free the encapsulated spermatozoa, allowing them to be moved into the common oviduct. Davey (1959) also suggested that the spermatophore was formed in the female during copulation when the spermatophore sac at the base of the aedeagus became everted into the vagina as it was being filled by the male secretions. When the male withdrew his aedeagus to terminate copulation, the spermatophore sac must have ruptured leaving the spermatophore behind. This early description of the transfer of sperm in *R. prolixus* prompted Scudder (1971) to categorize Triatominae into an evolutionary stage which employs an encased spermatophore.

Contrary to these previous reports, we have made two pivotal observations on living preparations that challenge the existence of a spermatophore and cause us to rethink how the sperm is transferred from the male into the female. First, the highly folded cuticle in the aedeagus, which was thought to give rise to the spermatophore sac (see Davey 1959), does not form the flexible expanding bag needed to create this protruding sac. This cuticle cannot be stretched beyond the aedeagus without being torn. Rather than being a collapsed sac, it lines the lumen through which the secretions pass from the copulatory duct at the proximal end of the aedeagus to the gonopore at its distal end. Second, a stream of spermatozoa can be observed extending from the mass of spermatozoa into the common oviduct at the early stages of copulation, well before copulation has been completed (Fig. 8). Therefore, when the spermatozoa enter the vagina, they are not encapsulated, but are delivered free to the base of the common oviduct. They are not hindered from entering the common oviduct, and the vestibulum does not need to free the sperm from any spermatophore. Although the body of the aedeagus only extends about half the distance into the vagina (Fig. 8), the placement of the sperm at the mouth of the common oviduct can be readily explained. The sperm are delivered to the vagina first, and the wing-like lateral endosoma processes guide them towards the common oviduct. Then the copious amount of accessory reproductive gland secretions is excreted into the vagina to create the jelly-like mass that packs the spermatozoa up against the common oviduct.

These observations demonstrate that *R. prolixus* does not form a spermatophore and that the phylogenic history of Triatominae needs to be reevaluated. Scudder (1971) categorized sperm transfer in insects into five modes from least to most evolved. Based on the early descriptions for *R. prolixus*, Triatominae were categorized into mode II—'Spermatophore molded in the male organ after aedeagus enters the female bursa copulatrix'. Triatominae should now be placed into mode

IV—'Male accessory gland material ejected into the female, often after sperm, the material not encapsulating sperm but forming a mating plug'. It is this jelly-like mass which may serve as a mating plug that is ejected by the female within a few hours after mating; it is not the remnants of a spermatophore which encased the spermatozoa.

6 Egg Production Associated with Feeding

6.1 Characteristics of the Blood Meal

The ingestion of a single blood meal by the adult female results in a bout of egg production that continues for about 14–21 days (Davey et al. 1986). Uribe (1927) and Buxton (1930) noticed that it was the amount of food ingested, and not just the act of feeding that triggered egg production, and Friend et al. (1965) found a good correlation between the number of eggs made and the amount of blood taken as food. But it is more than the quantity of blood that affects egg production for it also depends on the quality of blood. Raising *Rhodnius* colonies on different types of blood affects how many eggs are made. Significantly more eggs are made using human or rabbit blood compared to chicken, sheep or horse blood (Gomes et al. 1990).

Interestingly, how the insect feeds also affects egg production. In the study noted above, Gomes et al. (1990) employed an artificial membrane through which the insects were fed, and the insects were never brought into contact with a living host. Chiang and Chiang (2010) found that feeding insects on rabbit blood through an artificial membrane reduces the fecundity compared to feeding the insects directly on a rabbit. Thus, the amount of eggs made following the ingestion of a blood meal depends on the source of blood and the manner by which it is obtained. These results indicate that the chemical nature of the blood and sensory factors associated with feeding are variables that need to be taken into account. This situation also provides an opportunity to study how sensory input is integrated into the endocrine control of egg production.

6.2 Endocrine Control of Egg Production

Early studies on egg production in *R. prolixus* focused on the endocrine control showing that this process can be correlated with activity of the corpus allatum (CA), a secretory organ located in the retrocerebral complex at the midline where the paired corpora cardiaca (CC) join (Fig. 10). Wigglesworth (1934) identified the importance of this gland for maintaining the juvenile state. It releases juvenile hormone (JH) and is quiescent in the penultimate larval stage to permit metamorphosis

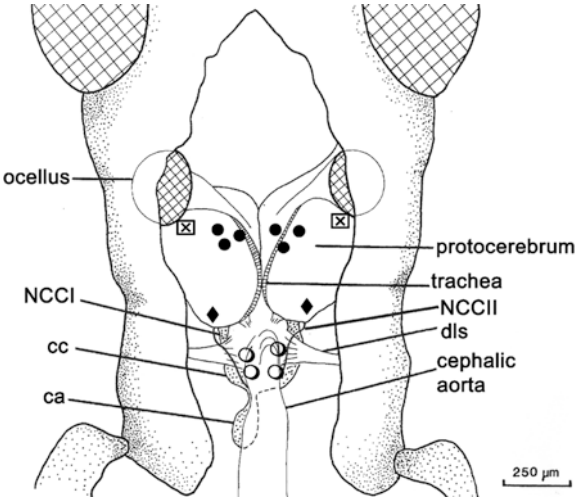

Fig. 10 Line diagram of the dorsal view of the brain and retrocerebral complex in *R. prolixus* illustrating the groups of cell bodies that are backfilled with cobalt applied to the neurohaemal site on the cephalic aorta. Cell bodies in the pars lateralis (X's) and the posterior margins of the protocerebral lobes (closed diamonds) send axons to the cephalic aorta via the nervus corpora cardiaca II (NCCII) which travels over the dorsal lateral supports (dls) of the aorta. Cell bodies in the pars intercerebalis (closed circles) send axons to the corpus cardiacum (cc) via the NCCI. ca, corpus allatum; open circles, cell bodies in the retrocerebral complex which send axons a short distance to the cephalic aorta. The location of cell bodies in the suboesophageal ganglion that are filled with cobalt applied to the cephalic aorta can be seen in Fig. 11. (Modified from Fig. 2 in Chiang and Davey 1988)

to occur. In the adult, decapitation posterior to the CA (Wigglesworth 1936) or surgical removal of the CA (allatectomy) in adult females after feeding reduces egg production (Davey 1967; Patchin and Davey 1968) and delays the maturation of the terminal oocytes (Pratt and Davey 1972).

It has been routinely assumed that JH is a gonadotropin in *R. prolixus* and that the number of eggs made reflects the amount of JH produced by the CA. When the head is severed behind the protocerebrum and in front of the CA, egg production increases significantly indicating that the brain imposes inhibition on the CA (Davey 1987a). Chiang (1998) discovered that the same increase in egg production is obtained simply by cutting the paired nervous corpus cardiaca IIs (NCCIIs). Yet this inhibition from the brain is not responsible for inhibiting the CA in the L5 to permit metamorphosis to occur. Cutting the NCCIIs prior to feeding L5 insects results in the formation of adults lacking their NCCIIs, and feeding these adults results in the same high levels of egg production when the NCCIIs are cut in the adults (Chiang 2000). This curious finding has been difficult to explain, especially since the identity of the JH in *R. prolixus* has remained unknown for several decades (Davey 2007). Fortunately, this gap in knowledge has recently been resolved; Villalobos-Sambucaro et al. (2020) have identified the JH in larval stages of *R. prolixus* to be

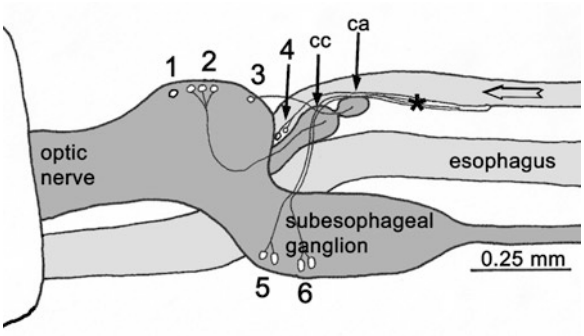

Fig. 11 Line diagram of a lateral view of the circulatory, nervous and digestive systems in the head of *R. prolixus* illustrating a possible vascular portal system in which several groups of neurosecretory cells terminate in the neurohemal site (asterisk) in the cephalic aorta. Open arrow represents direction of hemolymph flow through the lumen of the cephalic aorta. Groups 1 and 3 send axons through NCCII to the cephalic aorta, whereas group 2 send axons through NCCI to the corpora cardiacum (cc). Groups 5 and 6 in the suboesophageal ganglion send axons through the NCCIII to the cephalic aorta. ca, corpus allatum. For clarity, axons from group 1 have been omitted. (Modified from Fig. 9 in Chiang and Chiang 2017a)

JHIII skipped bisepoxide. It will now be possible to determine the role that this JH may play in reproduction.

Nonetheless, if the CA provides the endocrine link between feeding and egg production, and the activity of this gland is regulated by input from the brain, then sensory signals denoting the size of the blood meal could trigger egg production. In spite of being an attractive hypothesis, this mechanism initially lacked the nervous component to support it. The abdominal stretch receptors in *R. prolixus* only discharge in a phasic fashion and adapt quickly (Anwyl 1972). They could be responsible for the cessation of feeding, thus preventing gut rupture (Bennett-Clark 1963; Friend and Smith 1977), but egg production requires as much as 3 days postfeeding before it starts (Ruegg and Davey 1979). In addition, the mated female's ability to make more eggs than an unmated female was found to be related to the degree of distension of the crop during the egg production cycle following feeding (Davey et al. 1986). Such results suggested that a sensory receptor, which did not adapt, was needed to monitor the blood meal in the gut over long periods of time.

Without nervous receptors monitoring the size of the blood meal during egg production, hormonal factors were proposed to explain the differences between mated and unmated females. The most compelling factor was an antigonadotropin which reduced the effects of a gonadotropin on the vitellogenic follicles cells in the ovary. The source of the antigonadotropin was first attributed to the ovaries (Huebner and Davey 1973; Liu and Davey 1974), then to the abdominal neurosecretory organs located in each of the four full-sized abdominal segments (Davey and Kuster 1981; Kuster and Davey 1981). This antigonadotropin would be released in unmated females at about 7 days postfeeding to prevent JH from having its effect at the level of the follicle cells (Davey and Heubner 1974). This mechanism makes evolutionary

sense since the antigonadotropin would stop unmated insects from consuming their limited resources to make infertile eggs (Davey 1983). The existence of the antigonadotropin also explained why transecting the segmental nerves to the abdominal segments on one side of the animal resulted in a reduction in egg production (Davey 1982). If the release of the antigonadotropin from the abdominal neurosecretory organs is under neuronal inhibition, then transecting more of these nerves would result in more antigonadotropin released and a greater reduction in egg production. Recognizing that a synthetically produced antigonadotropin could be used as a chemical to deter egg production in domicile populations of Triatominae, considerable amounts of time and resources were invested to isolate this factor. Unfortunately, it has remained elusive (Davey 1987b, 2007).

As the hunt for the antigonadotropin started to wane, an abdominal pressure receptor capable of monitoring the quantity of the blood meal in the gut over extended periods of time was discovered (Chiang and Davey 1988; Chiang et al. 1990). These paired receptors are located in each of the full-sized abdominal segments in the body wall ventral to the gut, and the peristaltic movements of the gut would cause them to discharge. They fire tonically as long as a stimulus is applied. Being located in the body wall towards the midline and not to the side, they are ideally situated to monitor the size and movement of the gut over long periods of time. It is possible that input from these receptors, in response to the presence of the blood meal in the gut, initiates egg production 1–3 days after feeding. The axons of the abdominal pressure receptors travel from the abdomen into the mesothoracic ganglion along the same segmental nerves which serve the abdominal neurosecretory organs. The reduction in egg production correlated with the number of segmental nerves cut (Davey 1982) could reflect a loss of neural input from these pressure receptors, and also suggests that this sensory input up-regulates egg production. Schilman et al. (1996) have previously shown in *R. prolixus* that females laying eggs on feathers have a higher fecundity than females laying eggs on corrugated cardboard indicating the presence of sensory input which influences egg production.

6.3 Initiation by the Blood Meal

The blood meal in larval stages initiates the moult cycle by causing the release of a brain factor (prothoracicotropic hormone, PTTH) which turns on the activity of the prothoracic gland which, in turn, releases the moulting hormone, ecdysone (Wigglesworth 1974). In adults, egg production following the blood meal depends on the quality and quantity of blood, the interaction between the insect and a living host, and whether the insect is mated. But unlike what is known for the release of ecdysone, how the blood meal actually triggers and maintains egg production is still largely a mystery. It is possible that a hormonal signal originating in the abdomen of the fed insect is carried by the circulatory system to the brain to initiate activity of brain cells (e.g. see Mulye and Davey 1995), or these brain cells are activated by

nervous input from the abdominal pressure receptors monitoring the size of the blood meal in the gut (Chiang and Davey 1988).

But before suggesting how these hormonal and neuronal inputs could interact to initiate egg production, another caveat needs to be considered. The initiation of egg production following a blood meal depends on circulation of hemolymph through the cephalic aorta into the blood sinus under the brain. When circulation is stopped before or within a few hours after feeding, no eggs are made (Chiang and Davey 1990). This loss of circulation through the cephalic aorta occurs whenever the dorsal vessel is severed along its length anywhere from the mid-abdomen to the neck. It was not due to the inability of the nervous system to detect the size of the blood meal, nor the inability of substances to circulate outside of the dorsal vessel either to or from the head in the body cavity. In addition, the loss of circulation does not affect the ability of the insect to metabolize the blood meal indicating that the failure to make eggs is not due to a lack of nutrients supplied by the blood meal (Klingenberg and Chiang 2006).

These factors indicate that three systems, the nervous system, the endocrine system and the circulatory system, all interact to determine how feeding influences the number of eggs made. In mammals, these three systems come together in the vascular portal system of the hypothalamic pituitary axis (for a description, refer to Hadley and Levine 2007). In the mammalian system, neural input causes release of neurohormones from hypothalamic neurosecretory cells into the vascular portal system which carries them a short distance to the anterior pituitary where they influence the activity of endocrine cells. When Ruegg et al. (1982) and Orchard et al. (1983) first recorded electrical activity from the retrocerebral complex in *R. prolixus*, it was assumed that the retrocerebral complex only consisted of the CC and CA, and that neither structure was directly associated with circulation in the cephalic aorta. It is now known that the cephalic aorta is a major neurohaemal region from which neurosecretory cells can release neurohormones into the lumen of the cephalic aorta, and like the vascular portal system in mammals, these secretions can then be carried by the circulatory system. In *R. prolixus*, they will first travel directly into the head, and subsequently to the rest of the body.

7 Functional Anatomy of the Retrocerebral Complex

The first indication that the retrocerebral complex was more intricate and complex than previously thought was the discovery of spontaneous electrical potentials which were not coming from terminals in the CC (Chiang et al. 1989). These potentials were recorded from the base of the CA and along the aorta to the beginning of the thorax. The ultrastructure confirmed the electrophysiology. It showed the existence of a cephalic nerve which contained numerous axons and terminals possessing neurosecretory granules. The cephalic nerve is attached to the outside of the aorta, but as it travels posteriorly from the level of the CA, it becomes smaller, and a number of terminals appear on the lumen side of the aorta (Chiang et al. 1989).

Cobalt applied to the cephalic aorta backfilled to as many as six groups of cell bodies which could give rise to these terminals (Figs. 10 and 11). One group is located in the retrocerebral complex where the floor of the aorta joins with the top of the CC (group 4 in Fig. 11). The cell bodies in this group are found on either side of the aorta and their axons cross over before they extend posteriorly down the cephalic nerve. Two more sets of cell bodies are located in the ventroanterior region of the suboesophageal ganglion (SOG) (group 5 and 6 in Fig. 11). As their axons extend posteriorly, they converge into a single tract near the dorsal side of the SOG before they divide and ascend on either side of the cephalic aorta along the paired NCCIIIs. They also form elaborate arborizations in the SOG. Another two sets of bilaterally symmetrical cell bodies are located in the protocerebrum, one in the anterior dorsal region (group 1 in Fig. 11) and the other at the posterior points (group 3 in Fig. 11). Both groups of cells send their axons to the cephalic aorta through the tiny NCCIIs (Fig. 10). Until the cobalt backfilling mapped out this anatomy, only the large NCCI's were thought to carry axons of neurosecretory cells to the retrocerebral complex where they released their secretions from the CC directly into the body.

This functional anatomy allows the cephalic aorta in the retrocerebral complex to act like a vascular portal system (Fig. 11). Neurohormones released by cells into the cephalic aorta would be carried anteriorly to affect other cells and/or organs in the head or somewhere else in the body. The lack of egg production caused by stopping circulation through the cephalic aorta may be due to the secretions being trapped in the collapsed vessel. If so, one of these secretions could be a gonadotropin-releasing hormone, or possibly the gonadotropin itself. Examining the initiation and regulation of egg production in terms of this possible vascular portal system in *R. prolixus* provides a new way to explain old results and promises to be a rewarding venture.

8 Conclusion

This chapter crystallizes earlier research on sperm transfer and egg production in *R. prolixus*, and documents new insights into both processes. For sperm transfer, the aedeagus is designed to deposit sperm at the base of the common oviduct even before the male has finished placing his accessory gland secretions into the vagina. Having established the structure of the aedeagus, it will be possible to explore how it functions in sperm transfer, and to determine the role of the male accessory gland secretions in copulation. For the endocrine control of egg production, it is now known that the input from neurosecretory cells terminating in the neurohaemal site on the cephalic aorta needs to be considered. Since circulation through the cephalic aorta is also needed to trigger egg production, then these cells, which release secretions into the cephalic aorta, may be associated with an insect vascular portal system.

In the past 60+ years of research, our understanding of reproduction in *R. prolixus* has flourished. Today, with many of the questions surrounding the structure

and function of various organs being answered, the stage is set to explore anew the important process of sexual reproduction in vectors of Chagas disease.

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The Immune System of Triatomines

Nicolás Salcedo-Porras and Carl Lowenberger

Abstract All insects rely on an innate immune system to eliminate potentially lethal parasites and pathogens. In kissing bugs, this immune system comprises a plethora of elements that first recognize parasites and pathogens as non-self, followed by a multi-faceted response to eliminate them. There is an intriguing molecular interplay between kissing bugs and pathogens; the insects wish to eliminate pathogens while not eliminating the obligate intestinal microbial symbionts on which they depend for survival. Beneficial bacteria and the human parasite *Trypanosoma cruzi* survive in the lumen of the intestinal tract but are eliminated if they enter the hemocoel. The closely related parasite, *Trypanosoma rangeli*, however, survives in the hemocoel and later establishes in the salivary glands. Our understanding of triatomine-pathogen interactions has expanded dramatically due to the availability of new molecular and genetic tools that allow us to understand the expression and function of immune molecules, and the mechanisms by which they function. The immune system in triatomines has three main components: physical barriers, cellular responses (phagocytosis, nodulation, and encapsulation), and humoral factors (antimicrobial peptides, lectins, reactive oxygen and nitrogen species, and the phenoloxidase cascade). Here, we describe the current knowledge and adaptations of the innate immune system of triatomines, how it interacts with pathogens and parasites, and the challenges we face in studying these interactions.

Keywords Triatomine innate immunity · Toll · IMD · Host-parasite interactions · *Trypanosoma cruzi*

Abbreviations

AA	Arachidonic acid
AMG	Anterior midgut
AMP	Antimicrobial peptides
CLR	C-type lectin

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DAMP	Damage-associated molecular patterns
DTU	Discrete typing units
DUOX	Dual oxidase
ERK	Extracellular signal-regulated kinase
GI	Gastrointestinal
GIPL	Glycoinositolphospholipid
GNBP	Gram-negative binding proteins
Gr−	Gram-negative bacteria
Gr+	Gram-positive bacteria
IMD	Immune deficiency pathway or protein
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JNK	Jun N-terminal kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharides
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OUT	Operational taxonomic units
PAF	Platelet activating factor biosynthetic pathway
PAMP	Pathogen associated molecular patterns
PGRP	Peptidoglycan recognition receptors
PLA2	Phospholipase A2
PMG	Posterior midgut
PMM	Perimicrovillar membrane
PO	Phenoloxidase
PPO	Prophenoloxidase
PRR	Pattern recognition receptors
Pvr	Platelet-derived growth factor and vascular endothelial growth factor-receptor related
Pxt	Peroxinectin
RNS	Reactive nitrogen species
TNF	Tumor necrosis factor
TOR	Target of rapamycin

1 Introduction

A functional immune system relies, initially, on non-self-recognition, the ability of organisms to distinguish between their own tissues and those of other organisms. This principle is evident by the plethora of immune recognition and effector mechanisms identified in all organisms. Although certain immune molecules are used ubiquitously by many animals, each taxon has evolved strategies using different

groups of molecules and molecular pathways to achieve self and non-self-recognition. The study of innate immune responses can be traced back to the classic experiments by Elie Metchnikoff who demonstrated that starfishes and water fleas used phagocytosis to eliminate foreign bodies (Metchnikoff 1884; Metchnikoff and Freund 1884). Contemporary studies on vertebrate immunity by Paul Ehrlich revealed that specific receptors mediate the recognition of nutrients, self-tissues, and pathogens (Himmelweit 1958). These and other discoveries on the principles of innate and adaptive immunity resulted in Metchnikoff and Ehrlich being co-awarded the Nobel Prize in medicine and physiology in 1908 (Kaufmann 2008).

Invertebrates, including insects, depend exclusively on an innate immune system that is also present in vertebrates. This innate immune system is efficient at combating pathogens and parasites and is one of the most important factors that has allowed invertebrates to colonize almost every ecological niche on earth. While arthropods first appeared over 500 million years ago, and have diversified into many groups, our knowledge of their immune systems has focused historically on a narrow selection of species, usually bias towards holometabolous insects. These include the dipterans *Drosophila melanogaster,* due to its tractable genetics and our ability to generate and test mutants, and *Aedes aegypti* and *Anopheles gambiae,* due to their importance as vectors of parasites that cause significant diseases in humans. The lepidopterans *Samia cynthia*, *Hyalophora cecropia,* and *Galleria mellonella* have also served as models as they generate large amounts of hemolymph for protein characterization and histological studies. The study of these organism revealed a common set of immune elements that were assumed to be universal in all arthropods. Research on crayfishes, however, revealed the diversity of the arthropod immune response; crustaceans identify microorganisms using a different set of receptors and use modified signaling cascades compared with insects (Cerenius and Söderhäll 2018). More recently, *G. mellonella* has been used to study the evolution of fungus-insect interactions, revealing the role and participation of lipids, extracellular nucleic acids, and epigenetics in the invertebrate immune response (Whitten et al. 2004; Altincicek et al. 2008; Vilcinskas 2016). Furthermore, *G. mellonella* has been used as a model to identify new antibiotics and to study human microbial pathogenesis (Trevijano-Contador and Zaragoza 2018; Pereira et al. 2018; Cutuli et al. 2019). Despite many common factors, it is evident that each arthropod order, or even family, has made adaptations to the common core of immune elements (Benoit et al. 2016; Lai and Aboobaker 2017; Oliva Chávez et al. 2017; Zaidman-Rémy et al. 2018). Therefore, comparing common and unique immune adaptations may reveal important details of the evolutionary history of each taxon, and may provide us with information we can exploit to understand host-parasite interactions and reduce parasite transmission.

There are approximately 150 species of triatomines (see Chap 3). All triatomine nymphs and adults are obligate blood feeders of vertebrates, although phytophagy and hemolymph feeding also have been reported (Alves et al. 2011; Díaz-Albiter et al. 2016). Triatomines, especially *Rhodnius prolixus*, also have been used extensively for over 80 years to study basic principles of insect physiology (Wigglesworth 1933, 1972). The information from these classical studies using *R. prolixus* and

Triatoma infestans has now been expanded exponentially with complementary data generated from genome and transcriptome studies that have identified novel factors contributing to immunity and physiology (Assumpção et al. 2008, 2012; Bussacos et al. 2011; Medeiros et al. 2011; Ribeiro et al. 2012, 2014; Mesquita et al. 2015; Montandon et al. 2016; Ons et al. 2016; Calderón-Fernández et al. 2017; Hernández-Vargas et al. 2017; Latorre-Estivalis et al. 2017; Traverso et al. 2017; Brito et al. 2018; Nevoa et al. 2018; Santiago et al. 2018; Zumaya-Estrada et al. 2018).

Kissing bugs also have been studied extensively due to their role as vectors of parasites such as *Trypanosoma cruzi*, the causal agent of Chagas disease that currently infects ~8 million people and threatens 65 million more, almost exclusively in Latin America (Nguyen and Waseem 2018). As the insects feed on vertebrates, they ingest parasites as trypomastigotes. *Trypanosoma cruzi* develops exclusively within the lumen of the gastrointestinal (GI) tract of the insect where it transforms into epimastigotes and subsequently undergoes metacyclogenesis; the transformation from non-pathogenic epimastigotes to the infective metacyclic trypomastigote stages in the hind gut that are subsequently excreted with the feces. If *T. cruzi* moves into the hemocoel of the insect, it is killed by components of the innate immune system. A closely related trypanosome that is non-pathogenic to humans, *Trypanosoma rangeli*, is also acquired by the insects during blood feeding. This parasite penetrates the GI tract, migrates through the hemocoel where it transforms into epimastigotes, and then invades the salivary glands where the infective trypomastigotes multiply in anticipation of future transmission (Garcia et al. 2009; Azambuja et al. 2017). Thus, the same innate immune system must respond differently to very closely related parasites due to differences in their biology and transmission dynamics. Because triatomines have resident obligate microorganisms in their GI tracts on which they depend for survival, there must be mechanisms in place to recognize and eliminate potential pathogens while not also eliminating beneficial members of the gut microbiota. Understanding how triatomines regulate differential immune responses towards beneficial or pathogenic microorganisms has benefited extensively from the explosion of genome and transcriptome data. We can now compare and contrast innate immune responses across taxonomic groups to shed light on specific immune adaptations found commonly in the triatomines.

In the next sections we describe the current state of knowledge regarding the immune system of triatomines. This is arranged in three categories: (i) physical barriers, (ii) humoral responses, and (iii) cellular responses, but these are conceptual separations as elements within these categories may share molecules and pathways or interact synergistically and collaborate with each other to mount effective immune responses.

2 Physical Barriers, Cuticle Structure, and Wound Repair

In arthropods, physical barriers such as the cuticle, epithelial cells, peritrophic matrices, and perimicrovillar membranes are the first point of contact between hosts and pathogens. While the cuticle covers all tissues of ectodermal origin (external surfaces, the hindgut, and the foregut), epithelial surfaces of endodermal origin are found in the genitals, spiracles, the midgut, and below the cuticle. Most insects also have a transient peritrophic matrix/membrane that covers the midgut lumen. Triatomines lack a peritrophic matrix and instead possess a perimicrovillar membrane (PMM) with similar functions (Gutiérrez-Cabrera et al. 2016). All these structures are dynamic and respond to damage and invading pathogens.

2.1 Cuticle

Triatomines were among the first insects used to study the cuticle structure and later studies demonstrated the conservation of this structure across all arthropods (Wigglesworth 1933; Willis 2010; Parle et al. 2017). The cuticle plays an important role in mobility, prevention of desiccation, determining the body shape, and it acts as a barrier to pathogens (Moussian 2010). The cuticle consists mainly of chitin fibrils and a mixture of proteins, lipids, and waxes arranged in multiple layers; epicuticle, procuticle, and epidermal epithelial cells (Andersen 2010) (Fig. 14.1). The layers of the epicuticle that interact with potential pathogens have been described in detail (Wigglesworth 1947, 1988; Gordh and Headrick 2011). Most of the structural components of the epicuticle are produced in the fat body, hemocytes, and glandular cells and are secreted through the pore canals. In some insects the cuticle also has antimicrobial properties, but these have not been reported in triatomines (Butt et al. 2016; Qu and Wang 2018). Immediately below the epicuticle is the procuticle made of chitin fibrils and proteins that has antifungal properties (Wigglesworth 1933; Butt et al. 2016; Qu and Wang 2018). The epidermal epithelial cells, located at the base of the cuticle, generate the procuticle and epicuticle layers and are involved in wound repair (see Sect. 14.2.3) (Juárez and Fernández 2007). In addition to its other roles, the cuticle also may contribute to the tolerance or resistance to insecticide as pyrethroid-resistant triatomines have a thicker cuticle (Pedrini et al. 2009; Forlani et al. 2015; Mannino et al. 2018).

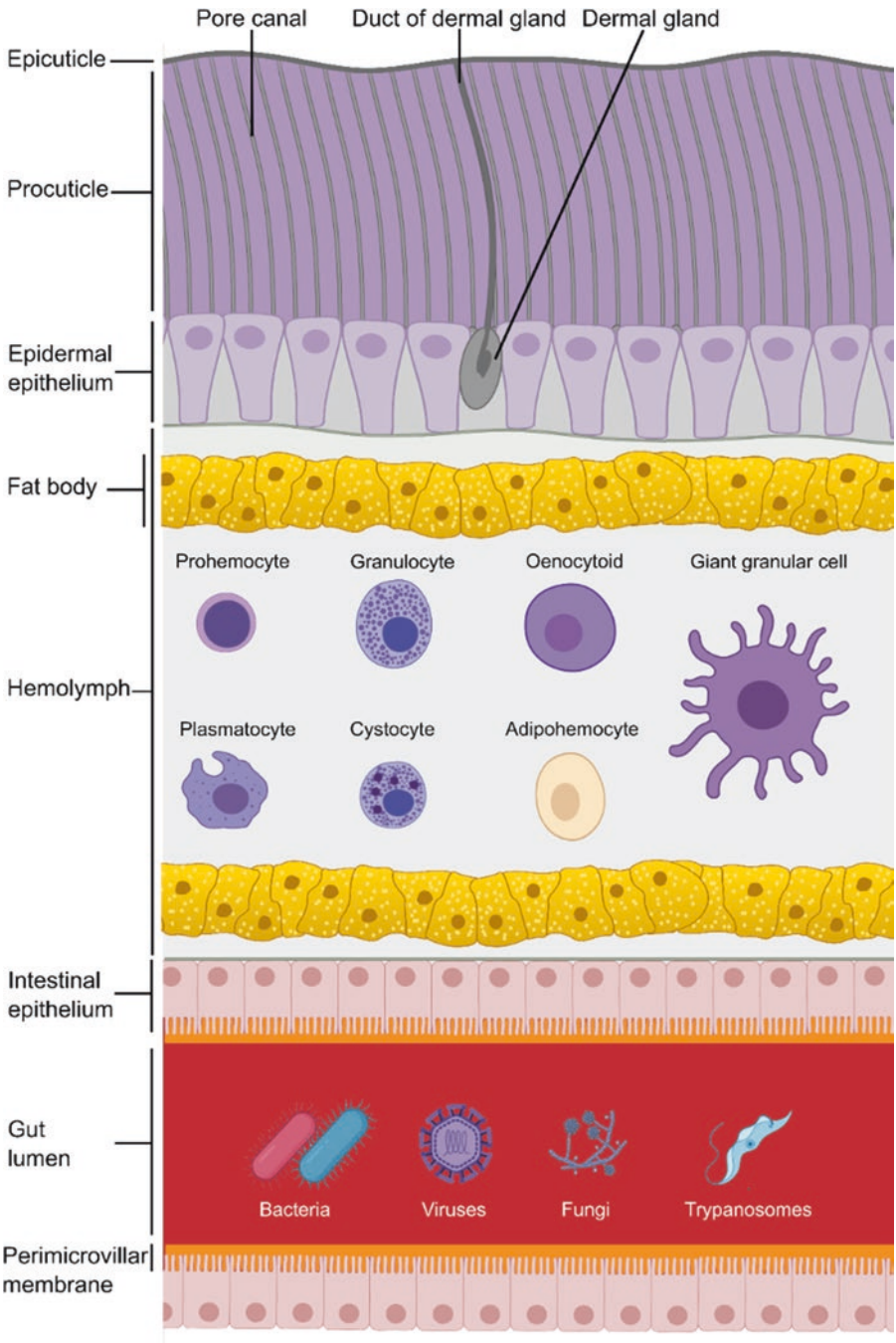

Fig. 14.1 Diagram representing the triatomine tissues and cell types that are involved in immune responses. The first barriers to pathogen invasion are physical structures including the cuticle, epidermal epithelia, intestinal epithelia, and perimicrovillar membranes. Once the physical barriers are breached the cellular immune response in the hemocoel is activated to fight pathogens. This immune response includes the activation of hemocytes, fat body cells, and epithelial cells. This also activates the humoral response which induces the expression of antimicrobial peptides as described in Fig. 14.2

2.2 Intestinal Epithelium and Perimicrovillar Membrane (PMM)

The intestinal epithelium is a single-cell layer that produces digestive enzymes, antimicrobial peptides (AMPs), and secretes a PMM into the luminal space (Gutiérrez-Cabrera et al. 2016). As an immune responsive organ the intestinal epithelium (Calderón-Fernández et al. 2017) expresses immune receptors and AMPs that eliminate ingested bacteria (Vieira et al. 2014, 2015, 2016; Zumaya-Estrada et al. 2018). The PMM protects the midgut epithelium from luminal digestive enzymes and pH changes, helps concentrate nutrients diluted in the blood meal, and protects against parasite invasion (Silva et al. 1995, 2004; Alves et al. 2007; Damasceno-Sá et al. 2007; Albuquerque-Cunha et al. 2009; Gutiérrez-Cabrera et al. 2016). The PMM also serves as an anchor to which *T. cruzi* and *T. rangeli* attach to multiply and complete their development (Garcia and Azambuja 1991; Kollien et al. 1998; Kollien and Schaub 2000; Azambuja et al. 2005; Alves et al. 2007), these interactions are mediated by lectins and sugars on trypanosomes and the PMM (Gutiérrez-Cabrera et al. 2014). Blocking PMM formation hinders *T. cruzi* development and adding PMM extract to an artificial diet or to parasite cultures in vitro restores *T. cruzi* development and induces metacyclogenesis (Burgos et al. 1989; Gonzalez et al. 1999; Carvalho-Moreira et al. 2003; Cortez et al. 2012; Dias et al. 2015). *Trypanosoma rangeli* invades the hemocoel more quickly in triatomines with a damaged PMM (Nogueira et al. 1997; Gomes et al. 2002; Gutiérrez-Cabrera et al. 2014), suggesting that while needed for parasite development, the PMM also serves as a physical barrier to parasites and pathogens invading the hemocoel.

2.3 Wound Repair

When the cuticle is breached, triatomines quickly activate repair mechanisms that involve cuticular, cellular, and humoral responses to close the wound, prevent the loss of hemolymph, entrap invading pathogens, and restore the cuticle (Lai-Fook 1968, 1970; Wigglesworth 1972; Parle et al. 2017). Our understanding of mechanisms underlying would repair are based on studies in dipterans and lepidopterans, in which a soft clot is formed initially at the wound by the degranulation of hemocytes to form a matrix that is cross-linked and hardened. Plasmatocytes then adhere to the clot and cuticle repair events continue (Lai-Fook 1968, 1970; Rowley and Ratcliffe 1976; Cerenius and Söderhäll 2011; Eleftherianos and Revenis 2011). This process involves components of the phenoloxidase (PO) pathways, hemolectins, transglutaminases, lipophorins, and hexamerins (Krautz et al. 2014). While few studies have described in detail the molecular mechanisms underlying clot formation in triatomines, most of the molecules used in other insects are present in the genome of *R. prolixus* (Mesquita et al. 2015; Zumaya-Estrada et al. 2018). After a clot is formed, the basement membrane contracts, hemocytes and oenocytoids are recruited, the area is melanized (Lai-Fook 1968, 1970; Krautz et al. 2014), and cuti-

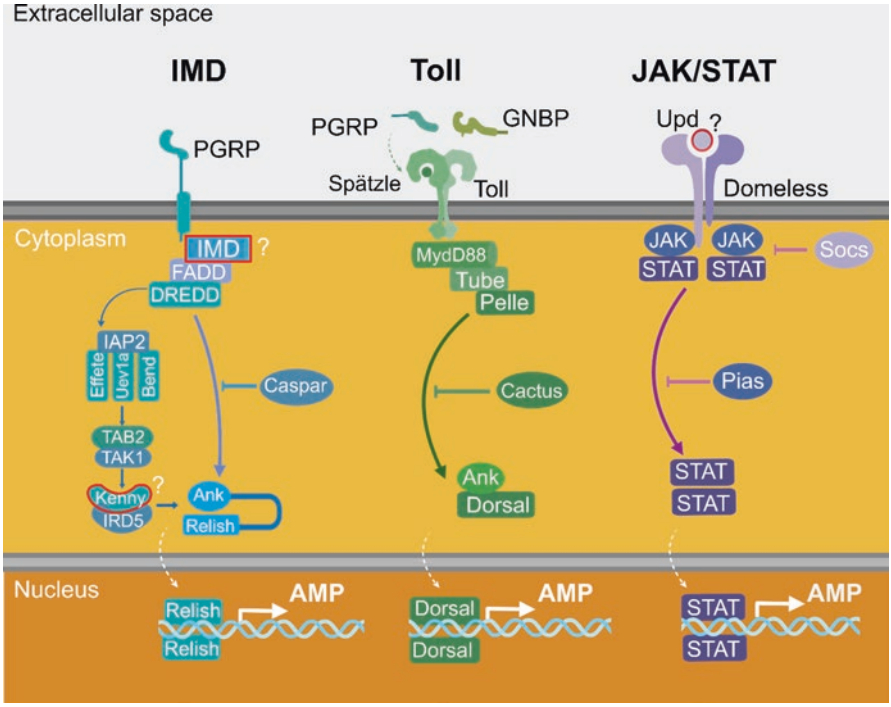

Fig. 14.2 The immune signaling pathways in triatomines. Triatomine orthologs, and their hypothetical interactions in the canonical IMD, Toll, and JAK/STAT pathways, are shown based on Zumaya-Estrada et al. (2018) and Salcedo-Porras et al. (2019). Orthologs outlined in red and denoted with a question mark have not been reported in triatomines. Arrows denote activation or induction while blunt-ended lines denote inhibition

cle restoration begins with the migration of peripheral epithelial cells and the deposition of the procuticle and basement membranes (Lai-Fook 1968, 1970). Studies on *Drosophila* sp. wound repair also indicate that the wound repair mechanism uses pathways involved in early ontogenesis and that there are common pathways and processes between insects and humans (Belacortu and Paricio 2011). Some of these conserved processes include the formation of a clot, the formation of an actin-cable, the migration of cells to the wound site, and the extension of surrounding epithelial cells (Rowley and Ratcliffe 1978; Belacortu and Paricio 2011), some of which have been reported in *R. prolixus* (Wigglesworth 1937).

Dozens of genes and pathways are modulated during the wound healing process. Describing all of these is beyond the scope of this chapter and only selected pathways that have been identified in triatomines will be discussed. Molecular studies of *Drosophila* sp. wound repair have characterized a set of 18 transcriptionally activated genes during wound healing (Juarez 2016). Most of these genes have orthologs in the wound repair system of other insects, including *R. prolixus,* as well as humans (Juarez 2016; Capilla et al. 2017). An extremely important pathway during

wound repair is the Jun N-terminal kinase (JNK) pathway which is activated rapidly upon wounding and acts as a master activator of the wound repair process in animals (Rämet et al. 2002; Lee et al. 2017; Tsai et al. 2018). Induction of the cytokine-activated Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway delineates the area of cell fusion at the edge of the wound (Lee et al. 2017; Tsai et al. 2018). Wound repair also activates the Toll (Patterson et al. 2013; Capilla et al. 2017) and immune deficiency (IMD) (Patterson et al. 2013) immune pathways even in the absence of an infection (see Sect. 14.3.1).

How immune related pathways are activated in the absence of microbial infection remains to be fully addressed. Some wound signaling molecules, known as damage-associated molecular patterns (DAMPs), have been proposed in insects. In this model, wounds induce a calcium influx that triggers the production of hydrogen by dual oxidase (DUOX), which in turn activates serine proteases, that activate the JNK, JAK/STAT, and Toll pathways (Capilla et al. 2017). Earlier studies in triatomines support this model, showing that the wound repair can be triggered by digested proteins coming from wounded cells, supporting the idea that this signaling is common in metazoans (Wigglesworth 1937). The presence of serine proteases can trigger wound repair in the absence of DUOX, and this can be mimicked by treatment with trypsin (Patterson et al. 2013). Both hydrogen peroxide and proteins cleaved by serine proteases are good candidates to act as DAMPs in triatomines.

Other pathways involved in the wound healing process of *Drosophila* sp. include the platelet-derived growth factor and vascular endothelial growth factor-receptor related (Pvr), the extracellular signal-regulated kinase (ERK), the target of rapamycin (TOR), the insulin, and the Hippo pathways (Tsai et al. 2018).

3 Humoral Immunity

The activation of immune responses and the molecular principles involved in non-self-recognition and elimination of foreign organisms are best demonstrated in insects by their humoral immune responses. This process involves three phases: recognition of the pathogen as non-self, signal transduction, and expression of effector molecules. First, membrane bound Pattern Recognition Receptors (PRRs) on insect cells recognize and bind to Pathogen Associated Molecular Patterns (PAMPs), common signature molecules found on the surface of many pathogens. For example, peptidoglycans and lipopolysaccharides on bacterial cell membranes are common PAMPs detected by plants, arthropods, and humans (Dzik 2010; Chen et al. 2014; Ranf 2016; Kagan 2017). Next, during the signal transduction phase the pathogen recognition is amplified through biochemical cascades that allow insects to mount an efficient response even to a low number of pathogens. These multistep cascades are tightly regulated to prevent an over-activation of the immune responses (Koshland et al. 1982; Pires-daSilva and Sommer 2003; Mobashir et al. 2012). The signal transduction pathways are highly conserved among very disparate organisms but may regulate different processes. For instance, the signal used in the IMD path-

way of insects to eliminate bacteria is almost identical to the tumor necrosis factor (TNF) pathway used by mammals to regulate cellular inflammation. Ultimately, the insect humoral responses result in the expression of many effector molecules including the broad arsenal of AMPs that kill pathogens directly.

3.1 Recognition and Signal Transduction

Most of our understanding of invertebrate humoral immune responses is based on antibacterial and antifungal responses in dipteran models such as *Drosophila* sp. (Valanne et al. 2011: Myllymäki et al. 2014). Insect PRRs such as the Gram-negative binding proteins (GNBPs), and peptidoglycan recognition receptors (PGRPs), recognize Gram-negative bacteria (Gr−), Gram-positive bacteria (Gr+), fungi, and potentially, protozoans and viruses (Hughes 2012; Zhang et al. 2012). These PRRs have diversified to detect different PAMPs; in *Drosophila* sp. some PGRPs exclusively detect Gr+ and others detect Gr− bacteria, activating different immune signaling pathways and inducing the production of appropriate effector AMPs (Valanne et al. 2011; Kleino and Silverman 2014; Myllymäki et al. 2014).

Once the non-self-recognition processes start, there is an activation of the principal immune signaling pathways that regulate AMP expression. In arthropods these are: (i) the IMD pathway, (ii) the Toll pathway, and to a lesser extent, (iii) the JAK/STAT pathway, all of which are activated by different PRR-PAMP interactions (Fig. 14.2). In *Drosophila* sp., the Toll pathway uses PGRPs to detect Lys-type peptidoglycans on the cell walls of most Gr+ bacteria, and GNBPs to detect polymers of D-glucose on the cell walls of fungi (Valanne et al. 2011; Lindsay and Wasserman 2014) (Fig. 14.2). The IMD pathway uses different PGRPs to recognize Dap-type peptidoglycans found on the cell walls of all Gr− bacteria and some Gr+ bacteria such as *Bacillus* sp. (Kleino and Silverman 2014; Myllymäki et al. 2014). Ultimately, pathogen recognition activates NF-κB transcription factors; Relish in the IMD pathway and Dorsal/Dif in the Toll pathway. These proteins are translocated to the nucleus where they induce AMP expression. The JAK/STAT pathway also regulates AMP expression, but its activation is indirect. This pathway responds to viral infections and inflammatory cytokines (West and Silverman 2018), and similar to Relish and Dorsal/Dif, the transcription factor STAT is translocated to the nucleus and regulates the expression of immune related genes (Morin-Poulard et al. 2013).

Triatomines have most of the genes involved in these three canonical dipteran immune pathways (Assumpção et al. 2008, 2012; Bussacos et al. 2011; Medeiros et al. 2011; Ribeiro et al. 2012, 2014; Mesquita et al. 2015; Montandon et al. 2016; Ons et al. 2016; Calderón-Fernández et al. 2017; Hernández-Vargas et al. 2017; Latorre-Estivalis et al. 2017; Traverso et al. 2017; Brito et al. 2018; Nevoa et al. 2018; Santiago et al. 2018; Zumaya-Estrada et al. 2018) (Fig. 14.2). Recent studies, however, have shown that triatomine responses towards pathogens are not identical to the dipteran models. The amino acid sequences of most of the genes from the

canonical Toll and JAK/STAT pathways are present in triatomines, are very well conserved, and are found easily by homology comparisons (Mesquita et al. 2015). The Toll and JAK/STAT pathways are functional in triatomines and we assume that the regulation is similar in dipterans and triatomines.

This is not the case, however, with the IMD pathway. In *R. prolixus*, the IMD pathway initially was reported to be absent, missing many genes, or non-functional compared with the canonical IMD pathway of dipterans (Mesquita et al. 2015). Recent data suggest that IMD pathway genes in *R. prolixus* are present but were not identified during automated genome annotation, possibly due to sequence divergence and multiple short introns (Panfilio et al. 2017; Zumaya-Estrada et al. 2018; Salcedo-Porras et al. 2019). Subsequent analyses using BLAST and hidden Markov model profiles confirmed that most elements of the IMD pathway are, indeed, present in triatomines and only the genes encoding IMD and Kenny have not been reported. The IMD pathway is functional, as initially predicted, it responds more strongly towards Gr- bacteria, and there is crosstalk between the major immune pathways in *R. prolixus,* and presumably in other triatomines, to mount a robust immune response (Zumaya-Estrada et al. 2018; Salcedo-Porras et al. 2019).

Missing, non-functional, and interrupted IMD pathways also were reported in other hemimetabolous insects and crustaceans (Kirkness et al. 2010; Shelby 2013; Zhang et al. 2014; Arp et al. 2016; Benoit et al. 2016; Chen et al. 2016; Saha et al. 2017; Cerenius and Söderhäll 2018; Zumaya-Estrada et al. 2018). Recent data suggest that this pathway is more plastic than the Toll and JAK/STAT pathways. In spite of these anomalies, the IMD pathway actively regulates immune responses in triatomines, as knocking down the transcription factor Relish reduces the expression of IMD regulated AMPs (Mesquita et al. 2015; Vieira et al. 2018; Salcedo-Porras et al. 2019). These data now indicate that the canonical IMD pathway is conserved in both hemi- and holometabolous insects.

In triatomines, as in dipterans, the IMD pathway is activated by infection with Gr− bacteria. In *R. prolixus* however, the IMD pathway is also responsive to Gr+ bacteria (Salcedo-Porras et al. 2019). No functional evaluation of PRRs has been done in triatomines but experiments in other hemipterans suggest that a single PGRP can mediate immune responses against both Gr+ and Gr− bacteria (Nishide et al. 2019). This may be the result of a single PRR activating different pathways or the crosstalk and interactions between immune pathways during the signal transduction events.

3.2 Humoral Effector Molecules

The hallmark of insect humoral immune responses is the expression of effector AMPs (Bulet et al. 1999). Since their initial discovery in the 1980s (Hultmark et al. 1980; Steiner et al. 1981), more than 2500 AMPs have been described (http://aps.unmc.edu/AP/). Most AMPs are short cationic molecules, categorized by their structure; linear and amphipathic, α-helical peptides, cysteine stabilized, proline-

rich, or glycine rich peptides (Bulet and Stöcklin 2005). AMPs differ considerably in their efficacy against different targets (Gr+, Gr−, fungi, trypanosomes) and the mechanisms by which they kill microorganisms (Bulet and Stöcklin 2005). AMPs are highly conserved across all insect orders and are produced principally by cells in the fat body, epithelia, and by hemocytes (Hillyer 2016; Yakovlev et al. 2017). While AMPs are highly conserved, each taxon has evolved its own arsenal of AMPs, reducing or expanding numbers as required and relying on different, possibly synergistic, combinations of AMPs to eliminate pathogens. Triatomines express many AMPs including defensins, lysozymes, trialysins, prolixicins, attacins, and diptericins (Amino et al. 2002; Kollien et al. 2003; Lopez et al. 2003; Araújo et al. 2006, 2015; Ursic-Bedoya and Lowenberger 2007; Balczun et al. 2008; Ursic-Bedoya et al. 2008, 2011; Waniek et al. 2009, 2011; Ribeiro et al. 2012; Díaz-Garrido et al. 2018). The AMPs identified in different triatomines, their tissues of expression, and the experimental stimuli used to induce their expression can be found in Salcedo-Porras and Lowenberger 2019 and Salcedo-Porras et al. 2019.

The activity of individual triatomine-derived AMPs has been evaluated in vitro*;* defensins eliminate Gr− and Gr+ bacteria (Lopez et al. 2003), prolixicin eliminates Gr− bacteria but not *T. cruzi* (Ursic-Bedoya et al. 2011), and trialysin is active against *T. cruzi.* (Martins et al. 2006, 2008). When triatomines are challenged in vivo with Gr−, Gr+, fungi, or trypanosomes, a more complex scenario occurs in which multiple AMPs are activated simultaneously. The synergistic effects of AMPs have been studied in *Drosophila* sp., but there is little information on the synergistic in vivo effects of multiple triatomine AMPs acting on pathogenic bacteria, fungi, and trypanosomes while not eliminating their essential obligate microbial symbionts (Tanji et al. 2007; Hanson et al. 2019).

The expression of AMPs may be time or tissue specific (Lopez et al. 2003; Flores-Villegas et al. 2015; Díaz-Garrido et al. 2018). While *R. prolixus* lysozyme-B is expressed strongly in the fat body (Ursic-Bedoya et al. 2008), lysozyme-A is expressed principally in the midgut (Ursic-Bedoya et al. 2008), and prolixicin is expressed in the fat body and in the midgut (Ursic-Bedoya et al. 2011). The timing and duration of specific AMP expression also differs and may depend on the invading pathogen. Some AMPs are highly expressed 4–8 h after infection (Marti et al. 2005; Ursic-Bedoya and Lowenberger 2007; Ursic-Bedoya et al. 2008, 2011) while others are not expressed strongly until 24 h after infection with bacteria (Lopez et al. 2003; Ursic-Bedoya et al. 2008; Vieira et al. 2014, 2015, 2016) or until 7 days after an infection with fungi or trypanosomes (Azambuja and Garcia 1987; Ursic-Bedoya et al. 2008; Waniek et al. 2011; Vieira et al. 2014, 2015, 2016; Lobo et al. 2015; Azambuja et al. 2017). These delayed responses may coincide with the slower invasion and replication processes in specific tissues by fungi and trypanosomes.

3.3 Lectins

Lectins form a large group of ubiquitous proteins that contribute to humoral and cellular responses (Liu et al. 2015; Xia et al. 2018). The vast majority of lectins bind to carbohydrate residues found on the surface of epithelia, hemocytes, and parasites, and interact directly with trypanosomes (Jacobson and Doyle 1996; Liu et al. 2015; Xia et al. 2018). Signaling C-type lectin receptors (CLRs) are crucial in the initiation of immune responses to fungi, but less is known of their role during infections with bacteria, viruses, and parasites. CLRs also can induce endocytosis, phagocytosis, and antimicrobial responses (Hoving et al. 2014). Lectins have been identified from the GI tract, salivary glands, and hemolymph of triatomines (de Miranda Santos and Pereira 1984; Barracco and Loch 1988; Gomes et al. 1991; Gregório and Ratcliffe 1991b; Basseri et al. 2002; Hypša and Grubhoffer 1995; Ratcliffe et al. 1996; Grubhoffer et al. 1997; Gourbière et al. 2012). In *R. prolixus* there are at least five genes encoding Type-C lectins alone, and the number of lectins reported from different triatomine species varies (Zumaya-Estrada et al. 2018). Earlier studies on lectins focused on their biochemical properties and their ability to agglutinate cells and parasites (Pereira et al. 1981). It is, however, difficult to link newer gene and transcriptome data with older biochemical studies since few of these older studies provided protein sequences (Mello et al. 1999). Recent findings suggest that the agglutination process is mediated by lipoproteins from ingested blood plasma (Moreira et al. 2018).

The immune role of triatomine lectins is complex and depends on the interactions among lectins, tissues, bacterial symbionts, and trypanosomes (Garcia et al. 2010a, b). Nonetheless, triatomine lectins are strongly implicated in the successful development and survival of *T. cruzi* (Mello et al. 1995) and *T. rangeli* (Feder et al. 1999; Gomes et al. 1999; Mello et al. 1999; Basseri et al. 2002). The survival of *T. cruzi* in the anterior midgut (AMG) of *R. prolixus* is partially dependent on the agglutination and nodulation responses involving lectins (Mello et al. 1999; Díaz-Albiter et al. 2016; Moreira et al. 2018) and the parasite strain under study (Mello et al. 1995; Ratcliffe et al. 1996).

There are seven genotypes of *T. cruzi* referred as Discrete Typing Units I to VI (DTU TcI-VI) and TcBat (Marcili et al. 2009; Brenière et al. 2016) that contribute to vector-parasite compatibility. For instance, *T. cruzi* strains that are strongly agglutinated by lectins in the AMG (strain Dm28c (TcI) and to a lesser extent strain CL (TcIV)) survive, while strains that are not agglutinated (strain Y (TcII)) are lysed (Mello et al. 1995, 1996; Ratcliffe et al. 1996). If strains Dm28c and CL are inoculated into the hemolymph of *R. prolixus*, the former is strongly agglutinated and it is able to survive for days while the latter is weakly agglutinated and eliminated quickly (Mello et al. 1995, 1996). These data corroborate observations of natural *R. prolixus* infections with *T. cruzi.* The most common infection in *R. prolixus* is with TcI, then TcIV, and rarely with TcII (Brenière et al. 2016), suggesting a DTU specific adaptation to specific triatomine species. The mechanistic basis of this compatibility is not clear but may be mediated through *T. cruzi* glycoproteins (such as

mucins) and triatomine lectins. Each DTU has a signature mucin sequence that can be differentially recognized by triatomine lectins (Mello et al. 1996; Buscaglia et al. 2006; Urban et al. 2011; Cámara et al. 2019). As such, *T. cruzi* epimastigotes that overexpress mucins increase their attachment to the hind gut cuticle but this attachment can be inhibited by pre-exposing the insects to DTU specific carbohydrates (Figueiredo et al. 2000; Gonzalez et al. 2013; Cámara et al. 2019). These findings support the importance of glycoprotein-lectin interactions in trypanosome-triatomine compatibility.

The developmental stage of trypanosomes also may affect the expression of their surface carbohydrates (Zimmermann et al. 1987; Jacobson and Doyle 1996; Basseri et al. 2002) and subsequent immune recognition by hosts. In natural infections, short epimastigotes of *T. rangeli* invade the triatomine hemocoel and transform quickly into long epimastigotes (Mello et al. 1995) and only long forms successfully invade the salivary glands (Mello et al. 1995; Whitten et al. 2001). When short and long epimastigotes from the same strain are exposed to hemolymph extracts, only the latter survive (Mello et al. 1995, 1999). Short and long forms express different carbohydrates on their surface, and the long *T. rangeli* forms are agglutinated, but not lysed, in the hemolymph (Mello et al. 1999; Basseri et al. 2002). Long forms also interact with the salivary gland epithelia, whereas short forms do not (Mello et al. 1999), suggesting that these adaptations are designed to help the long forms survive host immune responses. Inhibition assays with different sugars showed that these interactions are dependent upon lectins (Mello et al. 1999).

High density lipoproteins (HDL) also may contribute to parasite agglutination and survival (Moreira et al. 2018). Incubating *T. cruzi* with HDL from mammalian blood caused parasite agglutination, and insects fed with erythrocytes, normal plasma, and purified HDL showed an increased population of epimastigotes compared with insects fed with only erythrocytes and normal plasma (Moreira et al. 2018). These data contribute to our understanding of trypanosome agglutination mechanisms (Mello et al. 1996) and confirm that agglutination is needed for *T. cruzi* to survive (Brener 1973; Cortez et al. 2002, 2012; Ferreira et al. 2016; Moreira et al. 2018). An open question is whether or not *T. rangeli* uses similar survival mechanisms.

3.4 Reactive Nitrogen and Oxygen Species

Nitric oxide (NO) is a ubiquitous molecule in animals involved in diverse processes such as central nervous system (CNS) communication, memory formation, neural development, vasodilation, and platelet anti-aggregation (Nappi et al. 2000; Ribeiro and Brehélin 2006; Settembrini et al. 2007; Sfara et al. 2008; Sfara et al. 2011). NO acts also as an immune secondary messenger and as an inducible immune effector with potent oxidative effects against pathogens (Whitten et al. 2001; Foley and O'Farrell 2003; Whitten et al. 2007; Ribeiro and Brehélin 2006; Wu et al. 2012). NO is generated by the activity of nitric oxide synthase (NOS), molecular oxygen,

and nicotinamide adenine dinucleotide phosphate (NADPH) on L-arginine (Nappi et al. 2004) and can be further oxidized to form other reactive nitrogen species (RNS) all of which have strong toxic effects against pathogens (Alderton et al. 2001; Ribeiro and Brehélin 2006).

In triatomines, infection with bacterial LPS, *T. cruzi*, or *T. rangeli* induces changes in NOS transcript expression and NO levels. These changes are probably immune modulation or effector responses by the triatomines to reduce the infections, or a strategy by the parasites to survive the triatomine immune response (Whitten et al. 2001, 2007; Moyetta et al. 2017). Although no PRR for bacterial LPS is known in triatomines, injecting LPS into the hemocoel of *R. prolixus* induces NOS and NO production in the fat body and hemocoel and feeding triatomines with lipopolysaccharides (LPS) increases levels of NOS in the AMG (Whitten et al. 2001, 2007; Castro et al. 2012). Infection with trypanosomes also affects NOS and NO levels. When *T. cruzi* is in the midgut, it induces NOS transcription in the AMG and hemocoel and increases NO levels in the AMG, PMG, hindgut, and hemolymph. This rise of NO and NOS levels is believed to be a barrier to prevent *T. cruzi* from entering the hemocoel. NO levels decrease when *T. cruzi* leaves the MG and establishes in the hindgut (Whitten et al. 2007; Castro et al. 2012). It is unclear if these reduced levels are parasite or host induced. Changes in NOS and NO levels seem to be independent of commensal bacteria, as antibiotic treated insects also have low midgut NO levels in late stages of infection (Castro et al. 2012). The bacterial symbiont *S. marcescens* can increase nitrogen availability by converting urea into ammonia (Garcia et al. 2010b; da Mota et al. 2018). Further studies are required to elucidate the interactions among triatomines, their microbiota, trypanosomes, and NO-immune responses.

During *T. rangeli* infections, when the parasite is in the midgut, NOS levels are reduced in the AMG, fat body, and hindgut, while NO levels rise in the AMG, PMG, hemolymph, and hindgut. This NOS reduction is thought to be caused by *T. rangeli* to permit subsequent invasion of the hemocoel, while increased NO levels are likely the triatomine immune response to the infection. Once *T. rangeli* is in the hemocoel NOS levels remain low in the AMG, fat body, and start decreasing in the salivary glands. Upon invasion of the salivary glands, NOS rises in the AMG, hindgut, and salivary glands. NO levels decrease in the AMG and PMG, following the effects of the previously reduced NOS levels in early and mid-infections (Whitten et al. 2001, 2007). These dynamic changes serve to aid *T. rangeli* survive in the vector. Inhibiting NOS with S-methyl isothiourea sulfate increases parasitemia and insect mortality in triatomines infected with *T. rangeli*, showing that NOS and NO are needed to fight *T. rangeli* (Whitten et al. 2001, 2007). The non-infective *T. rangeli* H14 strain has effects in *R. prolixus* similar to those seen in *T. cruzi* infections and the parasites are lysed in the midgut (Whitten et al. 2001). These data suggest that infective *T. rangeli* modulates the immune response of triatomines for its survival, and may use molecules such as trypanosome surface glycoinositolphospholipids (GIPL), that reduce NOS levels in the salivary glands (Gazos-Lopes et al. 2012). It is unknown if the parasites use GIPL to modulate NOS and NO in other tissues.

Reactive oxygen species (ROS) are produced in response to pathogen infections and in concert with RNS can eliminate invading microbes (Zhu et al. 1992; Garcia et al. 2010a). ROS are generated by the activity of the enzyme DUOX and superoxide dismutase, producing superoxide, hydrogen peroxide, and (with NO) peroxynitrite (Müller et al. 2008; Azambuja et al. 2017) all of which have antiparasitic effects. ROS are also produced during the digestion of hemoglobin (Finzi et al. 2004; Graça-Souza et al. 2006), and therefore trypanosomes are constantly exposed to strong oxidative agents in the intestinal tracts of triatomines. Although ROS can be used to eliminate pathogens, they also may trigger parasite development. An enhanced oxidative medium induces epimastigote multiplication, while a reduced oxidative environment produces low epimastigote numbers (Nogueira et al. 2015). ROS contribute to effective immune responses as seen in the hemocoel during phagocytosis and prophenoloxidase (PPO) mediated melanization (see Sect. 14.4). While infective *T. rangeli* strains induce PPO and superoxide levels, non-infective *T. rangeli* strains induce even higher ROS levels likely contributing to their elimination. Short forms of both infective and non-infective *T. rangeli* strains induce high ROS levels and are eliminated, suggesting that short forms that use oxidative signals to transform into long forms can survive and invade the salivary glands (Whitten et al. 2001). Changes in surface carbohydrates in long form epimastigotes, triggered by ROS, may also be involved in the immune evasion process and in the infection of salivary glands (see Sect. 14.3.2).

4 Cellular Immunity

Cellular immunity refers to the innate defense responses carried out, or mediated, by cells, especially hemocytes. The processes include phagocytosis, encapsulation, nodulation, melanization, and aggregation (Strand and Pech 1995; Gillespie et al. 1997). The separation of humoral and cellular responses is not a functional separation as many humoral factors affect and regulate the activity of hemocytes, and hemocytes produce humoral defense molecules (Strand 2008).

4.1 Hemocytes

Hemocytes in triatomines are found principally in the hemocoel where they contribute to multiple immune functions including wound repair and cellular responses (phagocytosis, nodulation, encapsulation, and microaggregation). They also produce components of the humoral response (ROS, RNS, PO, and AMPs) (Krautz et al. 2014; Dubovskiy et al. 2016; Hillyer 2016). Hemocyte classification in insects is highly variable; each order has different hemocyte types and nomenclature (Ribeiro and Brehélin 2006), but their contribution to immune responses is highly conserved. Hemocyte classification traditionally has been based on morphological

characteristics (Jones 1965; Price and Ratcliffe 1974; Azambuja et al. 1991a; Borges et al. 2008). Seven triatomine hemocyte types have been described (Fig. 14.1), but not all types have been found in all species. It is unclear if this is due to low hemocyte populations or an actual absence of certain types (Wigglesworth 1933; Jones 1965; Lai-Fook 1968, 1970; Barracco et al. 1987; Azambuja et al. 1991a; de Oliveira and de Souza 2003; Borges et al. 2008; Ruiz et al. 2015; Avendaño et al. 2017). Prohemocytes appear to be the precursors of other hemocyte types; they are small and circular with a nucleus that occupies most of the cell and are the only hemocytes found to undergo mitosis. Plasmatocytes are the most common hemocyte type in triatomines. They are involved in the phagocytosis of trypanosomes (de Oliveira and de Souza 2003), bacteria, and foreign bodies (Borges et al. 2008; Figueiredo et al. 2008a, b). They also contribute to microaggregation and nodulation. Plasmatocytes are variable in shape, contain small granules and have elongated membrane bound vesicles. Granulocytes are also numerous and together with plasmatocytes compose 85–90% of all hemocytes, and contribute to the microaggregation of bacteria. Morphologically, granulocytes are round with numerous small dense granules in their cytoplasm containing variable-shaped nuclei. Cystocytes have not been found in all triatomines, possibly due to their low abundance and fragility in vitro (Azambuja et al. 1991a). Morphologically they resemble granulocytes but have larger granules surrounding the nucleus. They are likely involved in coagulation responses. Oenocytoids have been described principally from *Rhodnius* sp. while their role is unknown, their membrane bound vesicles may contain PPO. Oenocytoids are oval shaped with a homogeneous cytoplasm, membrane bound vesicles, and small oval nuclei. Giant granular cells are the largest hemocyte type, and their function is unknown. They possess numerous pseudopodia and can phagocytize other hemocytes. The role of adipohemocytes has not been reported, but similar to fat body cells, adipohemocytes are large, irregular in shape, have small nuclei, and contain large lipid droplets and numerous glycogen particles in their cytoplasm (Wigglesworth 1933; Jones 1965; Lai-Fook 1968, 1970; Barracco et al. 1987; Azambuja et al. 1991a; de Oliveira and de Souza 2003; Borges et al. 2008; Ruiz et al. 2015; Avendaño et al. 2017) (Fig. 14.1).

4.2 Phagocytosis, Nodulation, Encapsulation, and Melanization

Cellular mediated immune responses in triatomines result in a modification of hemocyte populations and numbers. In infections with Gr− bacteria, Gr+ bacteria, latex beads, and *T. rangeli,* the population of prohemocytes decreases, while plasmatocyte and granulocyte populations increase (Takle 1988; de Oliveira and de Souza 2003; Borges et al. 2008). It is likely that prohemocyte populations decrease as they differentiate into plasmatocytes and granulocytes and other cell types are present in lower numbers and may have supporting secondary functions.

Phagocytosis may well be the most ancient cellular immune response in animals; individual hemocytes engulf microorganisms and foreign bodies into phagosomes that fuse with lysosomes, forming phagolysosomes in which the foreign bodies are destroyed. In triatomines, phagocytosis is performed mainly by plasmatocytes (Takle 1988; de Oliveira and de Souza 2003). Experiments in which *Escherichia coli* (Gr− bacteria) or *Staphylococcus aureus* (Gr+ bacteria) were injected into *R. prolixus* suggest that the insect's immune system can differentiate between these bacteria; *E. coli* was readily phagocytosed while *S. aureus* was predominantly melanized and nodulated (Borges et al. 2008). Other insects also mount differential immune responses against bacteria, and this distinction is not based on Gram designation (Hillyer et al. 2004). It is likely that the well characterized PRRs in humoral responses and lectins also discriminate among different bacteria and fungi in cellular responses, but the mechanisms used to recognize protozoans, parasitoids, and nematodes are poorly understood in insects (Dubovskiy et al. 2016). An important aspect for further investigation is the effect of infection dose on immune responses. Bacterial doses vary among studies and different doses may determine the degree to which different elements of the immune system are activated.

Multicellular immune responses are used when phagocytosis of individual pathogens is inefficient or not possible. This may occur in infections with large numbers of bacteria, fungi, and protozoans (nodulation) or against larger parasites such as nematodes and parasitoid eggs (encapsulation) (Dubovskiy et al. 2016; Hillyer 2016). In triatomines, nodules and capsules are formed by plasmatocytes and granulocytes and the melanization response is thought to be aided by cystocytes, oenocytoids, and epithelial cells (Gregório and Ratcliffe 1991a; Gomes et al. 2003; Lu et al. 2014; Hillyer 2016). Within the capsules and nodules, precursor molecules in the PPO cascade such as tyrosine are catalyzed in a series of reactions that produce ROS, quinones, cytotoxic free radicals, and melanin that ultimately kill the pathogens (Nappi et al. 1995). The transcriptomes and genomes of triatomines indicate that the PPO cascade components are similar to those found in other insects (Zumaya-Estrada et al. 2018). The intensity of this response depends on the tissue type (Genta et al. 2010) and species of triatomine (Gregório and Ratcliffe 1991a).

Melanization is a highly conserved immune response that quickly generates melanotic materials that can be used in immune responses to kill parasites directly, but also in other processes such as wound healing. Melanization can be triggered by cuticle damage or the recognition of PAMPs, and begins with a serine protease cascade that activates phenoloxidase (PO) from the inactive precursor prophenoloxidase (PPO) (Jiravanichpaisal et al. 2006; Cerenius et al. 2008; Hiruma and Riddiford 2009). PO oxidizes phenols into quinones, which ultimately generate melanin (Jiravanichpaisal et al. 2006; Cerenius et al. 2008; Hiruma and Riddiford 2009). Melanotic materials may be deposited directly onto parasites or on hemocytes that have encapsulated pathogens. Secondary cytotoxic products, including ROS, are released during this process that contribute to pathogen death (Vavricka et al. 2010), but due to the potential toxic effects of these secondary products, this response is tightly regulated (Sterkel and Oliveira 2017). PO pathway genes have been found in multiple tissues and cell types in triatomines (Azambuja et al. 1991a, b; Ribeiro et al. 2014; Zumaya-Estrada et al. 2018;

Sterkel et al. 2019). However, there are fewer serine proteases in triatomines, and in the Hemiptera in general, compared with other insects (Zumaya-Estrada et al. 2018).

4.3 Regulation of Cellular Responses

The regulation of cellular and humoral immune responses in triatomines is under the control of the eicosanoid and platelet activating factor biosynthetic pathway (PAF) and the endocrine system (Fig. 14.3). Eicosanoids in insects mediate multiple physiological processes such as Malpighian tubule function, ovarian follicle development, and immune responses (Kim et al. 2018). Eicosanoids have been found to regulate phagocytosis, nodule formation, melanization, microaggregation, ROS and NADPH oxidase (NOX) production, PPO secretion, cell migration, wound repair, and AMP secretion (Stanley et al. 2009; Kim et al. 2018; Stanley and Kim 2018).

In the eicosanoid pathway, phospholipids on the membranes of hemocytes and fat body cells are hydrolyzed by phospholipase A2 (PLA2) to produce arachidonic acid (AA) that is transformed by different oxygenases into eicosanoids and PAF. Peroxinectin (Pxt) generates prostaglandins, epoxidase generates epoxyeicosatrienoic acids, and lipoxygenase (LOX) generates lipoxins, hepolixins, leukotrienes, and hydroxy fatty acids. All of these molecules function as signaling molecules that can be detected by cellular membrane receptors (Stanley and Kim 2011, 2018). In triatomines the inhibition of eicosanoids and PAF reduces cellular immune responses (Garcia et al. 2004a, b; Machado et al. 2006; Figueiredo et al. 2008a). Phagocytic responses to bacteria and yeast can be eliminated by pre-exposing the insects to dexamethasone, an inhibitor of PLA2, or WEB2086, an inhibitor of PAF (Garcia et al. 2004b; Machado et al. 2006; Castro et al. 2009), resulting in high pathogen numbers and high insect mortality. Conversely, injecting AA or PAF-like molecules along with the pathogens rescues normal immune responses (Garcia et al. 2004b; Machado et al. 2006; Figueiredo et al. 2008a, b; Castro et al. 2009). These experiments indicate that AA derivatives are required for hemocyte activation, microaggregation, and the PPO cascade (Castro et al. 2009).

Parasites such as *T. rangeli* can manipulate the eicosanoid and PAF pathways to reduce or turn off phagocytosis, microaggregation, PPO activation, and NOX and NOS production (Coutinho and Nussenzweig 1952; Tobie 1970; Watkins 1971; Añez 1983; Takle 1988; de Oliveira and de Souza 2003; Figueiredo et al. 2006, 2008a). If these insects are pre-exposed to AA or PAF, immune responses are re-established, parasitemias are reduced, and insect survival increases (Garcia et al. 2004a; Azambuja et al. 2005, 2017; Figueiredo et al. 2008b). Pre-exposure to inhibitors of PLA2, Pxt, LOX, and PAF further increase parasitemias and insect mortality (Garcia et al. 2004a). The precise mechanisms that *T. rangeli* uses to reduce PAF and PLA2 are unknown.

The insect's endocrine system also modulates cellular responses. After blood feeding, prothoracicotropic hormone is released, stimulating the secretion of ecdysone that initiates the molting process (Wigglesworth 1972; Orchard and Steel

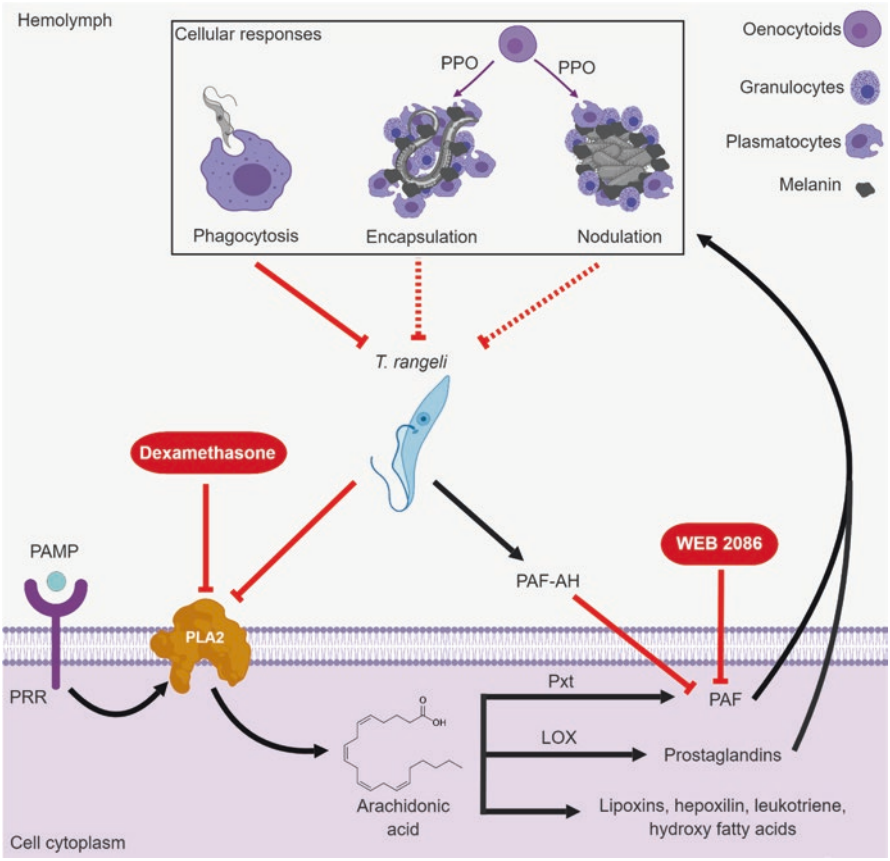

Fig. 14.3 Diagram representing the cellular responses and their regulation by the eicosanoid and PAF biosynthetic pathway during infection with *Trypanosoma rangeli*. Cellular immune responses are regulated by arachidonic acid derivatives. There is a direct relationship between prostaglandin and PAF levels with cellular responses. *Trypanosoma rangeli* manipulates PLA2 and PAF-HA levels to reduce cellular and PPO activity. Cellular responses regulated by the eicosanoid and PAF biosynthetic pathway are enclosed in the shaded box. Black arrows denote activation or induction, while red blunt-ended lines denote inhibition. Dashed lines denote hypothetical effects on *T. rangeli* due to the inhibition of PPO and cell proliferation

1980). Blood feeding also induces an increase in hemocyte numbers (Jones 1967; Ruiz et al. 2015). In experiments where triatomines are only fed with blood plasma or treated with a neuroendocrine antagonist, lower levels of prothoracicotropic hormone are released, fewer hemocytes are seen and cellular immune responses are diminished (Azambuja et al. 1991b, 1997). The normal immune responses can be rescued if the plasma is supplemented with ecdysone.

5 Triatomines and Microbiota

Triatomines have associations with specific obligate intestinal bacteria that are essential for their development and survival, which is not the case for most holometabolous insects (Ferrari and Vavre 2011). When triatomine nymphs emerge from the eggs they are sterile and acquire commensal bacteria from the feces of adult conspecifics. Early experiments demonstrated that insects raised in sterile environments (apo-symbiotic) had reduced development and lifespans (Brecher and Wigglesworth 1944; Auden 1974; Durvasula et al. 1999). The exact contribution of these symbionts to triatomines is still unclear. Supplementing blood meals with B complex vitamins restored the normal development of apo-symbiotic nymphs suggesting that these compounds were provided to the triatomines by the symbionts (Schaub and Eichler 1998). This artificial supplementation, however, precedes the time period, 24–48 h post blood feeding, when bacterial populations normally increase and it is unclear if this vitamin supplementation mimics the natural nutrient contribution from the microbiota. Triatomines containing bacteria that are auxotrophic to B complex vitamins also developed normally, suggesting that other nutrients may be essential for insect development (Hill et al. 1976), but these mutants had leaky phenotypes, and better mutant strains are needed to confirm these results. The diverse microbiota found in triatomine species also suggests a more general nutritional contribution from bacterial symbionts to the development of triatomines (Beard et al. 2002).

The principal commensals in *R. prolixus* are *Rhodococcus rhodnii* (Gr+ bacteria) and *Serratia marcescens* (Gr− bacteria). While initially it was thought that the association between a triatomine and its symbiont was rigid (Brecher and Wigglesworth 1944; Auden 1974), recent microbiome studies indicate that triatomine populations have much more flexibility in their association with bacterial symbionts than previously thought (Yassin 2005; da Mota et al. 2012, 2018; Gumiel et al. 2015; Lima et al. 2018; Montoya-Porras et al. 2018; Oliveira et al. 2018; Rodríguez-Ruano et al. 2018). Despite this flexibility, triatomines have a very low species richness in their microbiomes. As few as 20 operational taxonomic units (OTU) are recovered from the gut, demonstrating a strong selection for specific bacterial groups. The mechanisms by which triatomines maintain the network of microorganisms in their GI tracts are not clear. There must be a fine balance between the digestive and immune activities of triatomines that allow for normal physiological processes to take place, but which do not eliminate obligate symbionts in the GI tract.

The association of hemipterans with obligate symbionts is thought to have arisen in insects with a specialized and nutrient deficient diet, such as plant sap or blood. Some hemipteran taxa rely on intracellular bacteria to supply dietary deficiencies (Moran et al. 2005), and these primary symbionts are transmitted vertically (Jiménez-Cortés et al. 2018). Specialized structures to isolate symbionts from the immune system of their hosts have also arisen (Zaidman-Rémy et al. 2018). Insects in the suborder Heteroptera, including the triatomines, appear to have lost any primary symbiont associations and, instead, have adopted associations with extracel-

lular symbionts (Gordon et al. 2016). The number of microbes in the gut increases significantly after blood feeding and returns to basal levels within a few days (Azambuja et al. 2004), but there is no evidence that they are affected directly by digestive or immune factors. Furthermore, no tolerance mechanisms have been reported that would favor the development of obligate symbionts. The study of the molecular interactions between the digestive and immune systems of the insect, obligate and opportunistic microbes, and parasitic trypanosomes presents an opportunity to answer basic questions about the biology of this group of insects.

While bacteria and parasites have been studied in detail in the triatomines, the study of viruses has lagged behind. The only reported triatomine virus is *Triatoma* virus (TrV) isolated from *T. infestans* (Muscio et al. 1987). Very little is known of the pathogenic effects of this virus on the insects, its mode of transmission, or how it interacts with the innate immune system of its hosts (Muscio et al. 2000; Johnson 2015). TrV infections are reported to facilitate the establishment of *T. cruzi* in the insect gut (Marti et al. 2017) suggesting there may be immune-mediated virus-host interactions. The discovery of unstudied viruses offers exciting research opportunities to better understand the dynamics of multitrophic interactions among triatomines, trypanosomes, viruses, and bacteria.

Numerous entomopathogenic fungi have been reported from triatomines and some, such as *Beauveria bassiana,* are used as biocontrol agents. Fungi secrete hydrocarbon-degrading enzymes to breach the cuticle and express immunosuppressive compounds to inactivate the hosts' immune system (Napolitano and Juárez 1997; Juárez et al. 2000; Pedrini 2018). Triatomines respond to fungal invasion by expressing prophenoloxidase, hemolectins, and defensins, but these are inefficient once the fungi have established (Pedrini et al. 2009; Forlani et al. 2015; Lobo et al. 2015; Mannino et al. 2018). Thicker cuticles, demonstrated in pyrethroid resistant triatomines, do not seem to provide extra protection against *B. bassiana* (Pedrini et al. 2009; Forlani et al. 2015; Mannino et al. 2018). Other species of fungi including *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp., *Penicillium* sp., and *Verticillium* sp., have been identified from the GI tracts of field collected triatomines, but there are few data on how these fungi were acquired, if they negatively affect the insects, and if they are recognized in the GI tracts and activate an immune response (Moraes et al. 2001).

6 Triatomines and Trypanosomes

Triatomines are infected with the trypanosomes *T. cruzi* and *T. rangeli* when they feed on the blood of an infected host. Each of these parasites has similar, yet different, tissue-specific interactions that likely trigger common and tissue specific immune responses. *Trypanosoma cruzi* remains in the GI tract of the insect vector while *T. rangeli* moves into the hemocoel. The seclusion of *T. cruzi* in the GI track limits its interactions with host tissues to the midgut epithelia, PMM, and hindgut cuticle (Tyler and Engman 2001). Attachment to the gut epithelia, and parasite

development, requires the expression of the cysteine peptidase, cruzipain. Inhibiting cruzipain arrests metacyclogenesis (Uehara et al. 2012). Interactions with the PMM, mediated through lectins, are also required for *T. cruzi* development and survival (de Miranda Santos and Pereira 1984; Gomes et al. 1991; Jacobson and Doyle 1996; Mello et al. 1996, 1999; Brosson et al. 2017; Xia et al. 2018) (see Sect. 14.3.3).

Infection with *T. cruzi* also triggers changes in humoral immune responses and the populations and species richness of the gut microbiome. Many of these responses depend on the genotype of *T. cruzi* and the triatomine species (Mello et al. 1995; Ratcliffe et al. 1996). Specific DTU-triatomine combinations result in successful *T. cruzi* infections while others do not. The *T. cruzi* Dm28c strain (TcI) develops in *R. prolixus* and infection reduces gut bacterial populations, and increases antimicrobial activity, PPO levels, and AMP expression (Azambuja et al. 2004; Castro et al. 2012; Vieira et al. 2016). In *R. prolixus*, the Y strain (TcII), which does not establish in *R. prolixus*, does not significantly alter gut bacterial populations, does not stimulate AMP expression significantly, and is rapidly lysed (Azambuja et al. 2004; Castro et al. 2012; Vieira et al. 2016). *Trypanosoma cruzi*, also interacts with the cuticle. In the latter stages of metacyclogenesis infective trypomastigotes adhere to the wax layer in the insect's hindgut (Kleffmann et al. 1998). It is still unclear if trypomastigote populations are reduced when the insects molt and shed the cuticle and if repopulation occurs from midgut forms or from trypomastigotes already in the rectum (Kleffmann et al. 1998; Schmidt et al. 1998). Parasite-vector interactions mediated through the immune system may have more complex and indirect effects; fungal populations decrease in insects infected with *T. cruzi* (Lage-Moraes et al. 2001). Whether this is a result of the parasite activating antifungal components of the immune system, or direct competition between parasite and fungus, is unknown.

Natural salivary gland infections with *T. rangeli* are limited to members of the genus *Rhodnius*. *Trypanosoma rangeli* is also ingested during blood meals from infected vertebrates but, in contrast to *T. cruzi* that resides exclusively in the insect gut, *T. rangeli* penetrates the gut epithelium and migrates through the hemocoel into the salivary glands. *Trypanosoma rangeli* transforms into epimastigotes in the anterior midgut, enters the hemocoel, and then transforms into infective trypomastigotes in the salivary glands (Garcia et al. 2009; Ferreira et al. 2018). The capacity to survive immune responses in the hemocoels is an exclusive adaptation of *T. rangeli*; if *T. cruzi* is inoculated directly into the hemocoel, all parasites are eliminated (Pereira et al. 1981; Mello et al. 1995, 1996; Ratcliffe et al. 1996). Both trypanosomes activate cellular and humoral responses in the hemocoel (Ursic-Bedoya et al. 2011), but only *T. rangeli* survives in this location. Some of the strategies used by *T. rangeli* to survive include manipulating the eicosanoid and PAF cascades (see Sect. 14.4.3) and expressing different surface glycoconjugates (see Sect. 14.3.3) (Mello et al. 1999). AMP expression is also increased during *T. rangeli* infections, but this does not seem to influence parasite survival (Mello et al. 1995).

7 Conclusions

Our cumulative knowledge of triatomine immunity has revealed the presence of predicted and highly conserved elements of arthropod immunity and has identified many areas we have yet to study. It is unclear how non-self-recognition mechanisms, mediated through PRR-PAMP interactions, differ from holometabolous insects, how downstream signal transduction cascades are activated, and how single PGRPs activate multiple immune pathways. The newly available transcriptome and genome databases will allow us to take a comparative approach to dig deeper into specific aspects of triatomine immune responses. We need to clarify hemocyte classification, the role of hemocytes in producing humoral elements such as PPO and AMPs, and how the cellular and humoral arms of the immune response communicate with each other.

There is a lack of information on what fungi and viruses infect triatomines and how the hosts recognize and protect themselves from these pathogens. Understanding how triatomines eliminate these pathogens might help develop new approaches, and biological control agents, that circumvent the immune responses of the triatomines.

Understanding the intricacies of the complex triatomine-trypanosome-microbiome interactions requires a truly multidisciplinary approach using expertise in ecology, parasitology, microbiology, genomics, bioinformatics, biochemistry, and general biology to explain the multitude of interactions among these organisms. These approaches could establish triatomines as the model organism to study evolutionary aspects of innate immunity and to identify aspects we might exploit to reduce parasite transmission.

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Interaction of Triatomines with Their Bacterial Microbiota and Trypanosomes

Alessandra A. Guarneri and Günter A. Schaub

Abstract In this book chapter, we discuss the interactions between triatomines, two heteroxenous trypanosomatids—*Trypanosoma cruzi*, the etiologic agent of Chagas disease, and *Trypanosoma rangeli*, a sister species that is harmless to humans—and the diverse intestinal microbiota of triatomines, which include mutualistic symbionts. In order to colonize and proliferate, both species of trypanosomatids and their symbionts must survive the varying conditions in the different regions of the triatomine intestine. *Trypanosoma cruzi* multiplies mainly within the posterior midgut and in the rectum, where the infectious metacyclic trypomastigotes develop. In contrast, *T. rangeli* colonizes the whole intestinal tract, but predominantly the midgut, and subsequently invades and multiplies within the hemocoel, and then the salivary glands where metacyclogenesis occurs. The effect of triatomines on trypanosomes is evident in the differing susceptibility and refractoriness of different species/strains of triatomine to trypanosome infection. The diverse conditions in different regions of the triatomine intestine induce the development of specific forms of the trypanosomatids, which are also affected by the nutritional status of their triatomine vector, that is, by feeding and starvation. Reciprocally, the effect of the trypanosomatids on their triatomine vectors is strong in some *T. rangeli*-*Rhodnius* combinations, but is dependent on stressful conditions in *T. cruzi* infections of triatomines. Ingestion of blood-stage trypomastigotes induces intestinal humoral immunity, which in turn modifies the populations of bacteria present in the triatomine intestinal tract.

Keywords Triatomines · *Trypanosoma cruzi* · *Trypanosoma rangeli* · Microbiota

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1 Introduction

Triatomines can be infected with two different heteroxenous species of trypanosomes, which also infect humans: *Trypanosoma cruzi*, the etiological agent of Chagas disease, and *Trypanosoma rangeli*. These parasites can also share the same individual mammalian hosts, resulting in either single or mixed infections in triatomines in areas where the two trypanosome species have overlapping distributions (Carcavallo et al. 1975; Vallejo et al. 1988; Ramirez et al. 2002; Villacís et al. 2015). *Trypanosoma rangeli* is not pathogenic to humans, but is of medical relevance because it shares part of its soluble antigenic epitopes with *T. cruzi*, resulting in serologic cross-reaction, which can mislead the diagnosis of Chagas disease (Guhl et al. 1985, 1987; de Moraes et al. 2008; Zingales 2018). When infecting the gut of triatomines, trypanosomes share the same space with the triatomine bacterial and fungal microbiota, and the interactions between them can be decisive for both the parasites and the bacteria. One of the major drawbacks in understanding trypanosome dynamics in the field is the lack of knowledge concerning the interactions between the trypanosomes, their insect vectors, and the microbiota that lives in the intestinal tract of the insect. In this chapter, we provide an overview of the development of trypanosomes and microbiota in triatomines, as well as the effects produced by the presence of these microorganisms on the triatomines themselves.

2 The Microbiota of Triatomines

Although the blood ingested by triatomines is generally sterile, different bacteria still colonize the intestinal tract of triatomines. These bacteria are presumably acquired by contact of the mouthparts—both before and after blood ingestion—with the skin of the vertebrate host, and swallowing of air before molting, but probably mainly by coprophagy. The latter behavior is performed after the nymph had ingested blood, as demonstrated by the transmission of homoxenous trypanosomatids (Schaub et al. 1989a). Acquisition of bacteria might also occur through contact of triatomines with their egg shells during and/or after eclosion.

Investigations of the microbiota of triatomines can be separated according to methodology: either culture-dependent or -independent molecular biological identification of the intestinal bacteria. In general, the classical microbiological approach of culture-dependent identification begins with plating the diluted intestinal contents on agar and incubating the plates, followed by classification of the bacteria colonies according to macro- and microscopic characteristics, Gram reactions, physiological tests (oxidase, catalase, oxidation/fermentation), and Api-systems. In the case of Actinomycetales, the order that includes all mutualistic symbionts of triatomines known to date, identification using mycolic acids and *16S ribosomal DNA* sequencing is also used (Schaub 2020). Some of these tests were originally designed for the identification of bacteria from humans or anthropogenic

environments, and are not optimal for the identification of bacteria from triatomines. Since triatomines from laboratory colonies acquire bacteria from other species reared in the same insectarium (summarized by Gumpert 1962), only specimens collected from the field can give an indication of the native microbiota. According to studies employing the methodology described above, up to 18 different bacterial isolates, including Actinomycetales belonging to nine genera, are present in the guts of *Panstrongylus megistus*, *Triatoma infestans,* and *Triatoma sordida,* although marked variation occurs between individuals from the same species (summarized by Schaub 2020). Also, diverse fungi are present in the digestive tract of triatomines (Moraes et al. 2001a, b).

The experimental procedure for the identification of mutualistic symbionts is, in principle, straightforward. After challenging axenic nymphs with an individual bacterial isolate, the development of the nymphs is followed up, and used to infer the mutualistic character of the bacteria. If the bacteria are able to colonize the triatomines, and the nymphs develop normally, then the bacterium is considered a mutualistic symbiont; if the nymphs show signs of abnormality, then the bacterial isolate is not considered to be a mutualistic symbiont. The major effects of aposymbiosis (i.e., removing obligate mutualistic symbionts) are retarded nymphal development, increased nymphal mortality rates, disturbances to digestion and excretion, and a failure to develop into adults (summarized by Eichler and Schaub 1998). However, this experimental procedure for determining the symbiotic character of bacteria is not easy to implement practically, especially with regard to axenic maintenance of triatomine nymphs. Axenic maintenance begins with disinfection of the surfaces of eggs followed by sterile feeding with pig blood from emergence up to the third nymphal instar (this blood enables a normal development). The third instar nymphs are then challenged with either a mixture of sheep blood and one of the bacterial isolates to be tested preferably Actinomycetales or uninfected sheep blood only as a control to determine the effect of the absence of the bacteria used to challenge the other triatomines. The subsequent nymphal instars and adults are then fed with sterile sheep blood (Eichler and Schaub 1998; Schaub 2020). After each blood-feeding, the feces of nymphs and adults are incubated on agar plates to confirm either the absence of bacterial infection in the control group (i.e., that they are aposymbiotic) or the presence of only the bacteria used to challenge the infected group (Eichler and Schaub 1998). Mutualistic symbionts must establish in the gut and enable normal nymphal development through the adult stage with low nymphal mortality rates (up to 10% during all nymphal instars), and adult reproduction and hatching rates of their progeny comparable to the rates of the laboratory colonies. These characteristics occur in infections of: *Rhodnius prolixus* with *Rhodococcus rhodnii*; *P. megistus* with an un-named *Rhodococcus equi*-like isolate and *T. infestans* with *Rhodococcus triatomae*, which was previously classified as a *Nocardia* sp. (Eichler and Schaub 2002), but later re-named as *R. triatomae* (Yassin 2005); and *T. sordida* with an un-named *Gordonia rubropertinctus*-like isolate (summarized by Schaub 2020). According to a survey of the microbiota of laboratory-reared adults, *Rhodnius ecuadoriensis* also seems to possess *R. rhodnii* as a mutualistic symbiont (Rodríguez et al. 2011). After identification of *R. rhodnii*—the mutualistic symbiont of the

triatomine *R. prolixus*—its function as a vitamin B supplier was postulated, because the fitness costs associated with the loss of *R. rhodnii* were reduced in aposymbiotic nymphs after feeding blood supplemented with either B vitamins or *R. rhodnii*. However, in investigations of the eight B complex vitamins, different vitamins have been suggested to be produced by *R. rhodnii* (summarized by Vallejo et al. 2009). Another argument against the vitamin B hypothesis is that when fed different auxotrophic mutants of *R. rhodnii*, which are each unable to synthesize particular B complex vitamins, triatomine nymphs develop normally (Hill et al. 1976).

Recently, a number of investigations have focused on culture-independent identification of the triatomine microbiota, including laboratory colonies of six different triatomine species (da Mota et al. 2012; Vieira et al. 2015; Díaz et al. 2016; Carels et al. 2017), as well as field collected specimens of *Triatoma brasiliensis*, *T. pseudomaculata*, *T. maculata*, *T. dimidiata*, *T. sanguisuga*, *T. protracta*, *T. sordida,* and *Rhodnius pallescens* (Gumiel et al. 2015; Montoya-Porras et al. 2018; Orantes et al. 2018; Rodríguez-Ruano et al. 2018; Oliveira et al. 2018). Overall, these investigations demonstrate that gut bacteria species composition varies by triatomine species. The intestinal tracts of *T. maculata* and *R. pallescens* are colonized by a low diversity of bacteria (Montoya-Porras et al. 2018), similar to that of triatomines from laboratory colonies (da Mota et al. 2012; Vieira et al. 2015; Díaz et al. 2016). However, a high diversity of bacteria is also possible—for example, field-derived posterior segments of the abdomen of *T. dimidiata* have about 500 bacterial species depending on the source of their blood meals (Dumonteil et al. 2018; Orantes et al. 2018). Sometimes field-derived triatomines are infected by *Serratia marcescens*, and often by the intracellular bacterium *Arsenophonus triatominarum*, as well as different actinomycetes. *Arsenophonus* is also regularly present in laboratory colonies of triatomines (Díaz et al. 2016). In some investigations, Actinomycetales were not found (da Mota et al. 2012; Orantes et al. 2018), which is a curious finding considering that only Actinomycetales have been identified as mutualistic symbionts of triatomines, as mentioned. However, in the laboratory, aposymbiotic triatomines subsequently infected with non-actinomycete bacteria develop into adults if they blood-feed on rodents or chickens (Schaub 2020).

With regard to ontogeny, the microbiota in field-collected *T. sordida* varies according to developmental stage: the relative proportions of Actinobacteria are reduced in older instar nymphs and adults (Oliveira et al. 2018). However, these data are based on apparently fully blood-engorged bugs of each nymphal instar and adults of which younger nymphs possess a higher digestion rate than older ones. Since the development of the mutualistic symbionts is affected by the period of time after blood meal ingestion (Eichler and Schaub 2002), this might have caused the changes in the relative proportions of Actinobacteria. In investigations of the microbiome of different nymphal instars and adults of laboratory colonies of *R. prolixus*, first instar nymphs possess the highest diversity of bacteria and the lowest proportion of *Rhodococcus* (Rodríguez-Ruano et al. 2018). During ontogeny, bacterial diversity decreases while the proportion of *Rhodococcus* increases markedly. However, a clear identification as *R. rhodnii* is missing. The decrease in bacterial diversity seems to be either a direct effect of *Rhodococcus* or an indirect

consequence of changes in intestinal immunity of the triatomine resulting in the killing of other bacteria. The increase in *Rhodococcus* might be due to the increasing number of opportunities for coprophagy, which occurs in each nymphal instar.

With regard to different regions of the intestinal tract, in field-collected *T. sordida* the microbiota in the three major regions of the gut differ, exhibiting either lower or higher abundances of bacteria from different genera (Oliveira et al. 2018). Determination of the abundance of mutualistic symbionts in axenically-reared *R. prolixus* and *T. infestans* at different times after experimental infection with them shows that the posterior midgut and rectum of unfed fifth instar nymphs contain 0.01–1.0% of that present in the anterior midgut (Eichler and Schaub 2002). The ingestion of blood reduces the density of mutualistic symbionts in the anterior midgut. However, within 7 days after blood ingestion, the density of these symbionts increases 80-fold. In contrast, in the posterior midgut and rectum, they remain at the low levels regardless of blood-feeding (Eichler and Schaub 2002).

3 Interactions of Triatomines with *T. cruzi*

3.1 The Parasite

Trypanosoma cruzi is the etiologic agent of Chagas disease, a neglected disease endemic to Latin America. In addition, Chagas disease is becoming relevant for other countries such as the United States of America, Canada, and some European countries, as people infected with *T. cruzi* immigrate to these countries (Coura 2015). Only mammals are vertebrate hosts of *T. cruzi*, which is mainly transmitted by triatomines. The populations of *T. cruzi* possess predominantly a clonal genetic structure, but genetic recombination can occur (Tomasini and Diosque 2015). Traditional classification based on enzyme electrophoresis recognizes three groups, named zymodemes (Z1 to Z3), while multilocus genotyping enables *T. cruzi* strains to now be classified into seven groups, with these discrete typing units being named TcI to TcVI and Tcbat (Zingales et al. 2009, 2012; Marcili et al. 2009). [In the following text, the *T. cruzi* groups mentioned are defined according to the DTU classification]. Within one typing unit, the biological characteristics of strains differ strongly, for example, multiplication rate during in vitro cultivation, virulence, and pathogenicity for laboratory mammals, or the multiplication rate and metacyclogenesis rate in vivo in the vector (summarized by Schaub et al. 2011). Different developmental stages occur in the mammalian host and the triatomine vector. In mammals, intracellular multiplying amastigotes develop into the non-replicative slender, stumpy or intermediate blood trypomastigotes. In the vector, these trypomastigotes transform into multiplying spheromastigotes and epimastigotes, which themselves develop into non-replicative metacyclic trypomastigotes, in which the kinetoplast is present in a more subterminal position than in blood trypomastigotes (Schaub et al. 2016).

3.2 *Development of* T. cruzi *in the Vector—Effects of the Vector on* T. cruzi

Similar to other vector-parasite systems, vector susceptibility and refractoriness are determined by a combination of the trypanosome strain and the species/strain of the triatomine (summarized by Noireau et al. 2009; Garcia et al. 2010a; Schaub et al. 2016). Probably, all species of triatomines are potential vectors of *T. cruzi* (Schofield 1994). The high level of human migration frequently introduces triatomines to new locations, where they are susceptible to the local strains of trypanosomes, although often less so than the local native vectors (e.g., Araújo et al. 2008; Noireau et al. 2009; Mejía-Jaramillo et al. 2009; Sandoval-Rodríguez et al. 2019). Infection of an introduced triatomine with a local strain of *T. cruzi*—or, conversely, of a local vector with an introduced *T. cruzi* strain—might be lost under adverse conditions, for example, during triatomine starvation, and this is perhaps the reason for the loss of *T. cruzi* infection from *T. dimidiata* (Vargas and Zeledón 1985). Depending on the geographic region, natural mixed infections with different strains of *T. cruzi* belonging to different discrete typing units can occur in the field (e.g., Fernandes et al. 1999; Cortez et al. 2006; Campos-Soto et al. 2016).

Trypanosoma cruzi colonizes the midgut, the rectum and both regions of the Malpighian tubules (the tubes themselves and the four rectal ampullae) (Schaub and Böker 1987; Schaub et al. 1989b; Ferreira et al. 2016; Schaub 2016). Since the development of trypanosomes in the cardia at the beginning of the anterior midgut, and in the Malpighian tubules including the rectal ampullae, have not been investigated in detail, we will focus here on trypanosome infection of the distensible part of the anterior midgut, the posterior midgut and the rectum. Investigations using epimastigotes for experimental infection seem to offer only limited information, especially with regard to studying the early stages of infection in the anterior midgut of individual triatomines. In the field, epimastigotes will only initiate an infection under natural conditions after coprophagy of infectious feces or after cannibalism resulting in the ingestion of the contents of the anterior midgut of the attacked triatomine (Schaub 1988a). During coprophagy, triatomines presumably ingest only low numbers of trypanosomes. However, investigations considering the posterior midgut several days after infection with epimastigotes are informative and likely to be representative of natural oral infections, because the trypanosome populations should be similar to those occurring after an infection with blood trypomastigotes.

Development of *T. cruzi* in the Anterior Midgut

After ingestion of infectious blood into the distensible anterior midgut, the blood trypomastigotes have to survive and differentiate in an environment in which the conditions differ strongly from those of the mammalian blood. Glucose is no longer abundant, and the parasite has to use amino acids and lipids for metabolism. In addition, movement is strongly reduced by the increasing concentration of blood cells

and the lysis of erythrocytes. This often results in a jelly-like consistency of the concentrated blood; the blood of guinea pigs crystallizes in the live animal (Bauer 1981; Lehane 2005).

During the initial period immediately after blood meal ingestion, salivary compounds from the triatomine are present in the intestinal contents, and additional anticoagulatory and antimicrobial compounds are secreted into the lumen by the wall of the anterior midgut (Müller et al. 2008; Meiser et al. 2010). In addition, the pH becomes increasingly acidic (Balczun et al. 2012b). None of these factors has been connected to specific developmental steps of trypanosomes in the anterior midgut, except interaction with the salivary gland-secreted antimicrobial peptide trialysin, produced by *T. infestans*, which lyses blood trypomastigotes (Amino et al. 2002). Trialysin is apparently neutralized by a compound secreted by *T. cruzi* epimastigotes (Kulkarni et al. 2013). Agglutinins and hemolysins are believed to determine the initial establishment of *T. cruzi* in the vector (summarized by Azambuja et al. 2005a, b, 2017). In *R. prolixus*, epimastigotes of two *T. cruzi* strains are agglutinated but not lysed, whereas those of another strain are not agglutinated but lysed (Mello et al. 1996).

The colonization of *T. cruzi* in the anterior midgut apparently depends on the triatomine species, as well as on the parasite strain. In a study evaluating the *T. cruzi* colonization process in *P. megistus*, the morphological transformations of the parasite are discrete; in the first days blood trypomastigotes and some intermediate forms are found. A residual population of rounded parasites that does not multiply occurs in established infections (Dias 1934). After infection of *T. infestans* by either the CL (TcVI), FL or MR *T. cruzi* strains, originally isolated from naturally-infected *T. infestans*, blood trypomastigotes transform into round and pear-shaped forms. A few days later, these forms apparently aggregate into large masses of rounded parasites, which seem to be fused in some cases, suggesting the possibility of DNA exchange (Brener 1972). In *R. prolixus* infected by either the CL, CL Brener (TcVI) or Dm28c (TcI) strains, a strong reduction in the number of blood trypomastigotes occurs in the first 24 h after ingestion (Dias et al. 2015; Ferreira et al. 2016), and no parasites are found after 96 h in fresh examinations (Ferreira et al. 2016). Studies using qPCR have found the presence of a residual population of a few dozen parasites that does not increase after blood-feeding (Dias et al. 2015; Ferreira et al. 2016).

Epimastigotes develop only in the posterior midgut and rectum (Ferreira et al. 2016). When incubating blood trypomastigotes of the *T. cruzi* strain CL in vitro with extracts of the anterior midguts of either unfed or recently blood-fed *R. prolixus*, only the extracts of recently fed bugs induce significantly higher parasite mortalities (Ferreira et al. 2016). A recent study that evaluated the infectivity to mammalian hosts of recently differentiated epimastigotes of the Dm28c strain also found a reduction in the numbers of parasites in the anterior midgut of *R. prolixus* a few hours after infection (Kessler et al. 2017). Blood trypomastigotes were seen differentiating into amastigote-like forms a few hours after arriving in the anterior midgut. These forms agglutinated after 1 or 2 days of infection, and at 5 days after infection they showed a more elongated body. No epimastigotes were seen in this intestinal region at 5 days post-infection (Kessler et al. 2017). However, in *T.*

infestans infected by the Chile 5 (TcI) and 7 strains of *T. cruzi*, which originate from the same village in Chile as the triatomine colony studied, epimastigotes were present in the anterior midgut of all nymphs after molting (Schaub 1988a). If the nymphs subsequently feed on chickens or mice, the epimastigotes in the anterior midgut are either killed or remain alive, respectively, but this does not strongly affect the development of *T. cruzi* in the posterior midgut and rectum (Schaub 1989a).

Development of *T. cruzi* in the Posterior Midgut

After passage of amastigotes/intermediate forms from the anterior midgut into the posterior midgut, the *T. cruzi* epimastigotes develop and multiply (Schaub 1989a). A depletion of glucose is believed to induce the transformation of amastigotes into epimastigotes (Tyler and Engman 2000, 2001). The development of trypanosomes in this intestinal region has been investigated in detail using a system in which vector and parasite—*T. infestans* and the *T. cruzi* strains Chile 5 (zymodeme 1, TcI) and Chile 7 (zymodeme 2, no DTU classification)—are originated from the same village in Chile. In this research, the parasites were maintained since 1983 either in storage at −80 °C or cyclically passaged through mice and triatomine vectors (Böker and Schaub 1984). At 1 week post-infection (p.i.) of second instar nymphs, amastigotes, spheromastigotes, and many intermediate stages occur (Schaub 1989a). After an initial uptake of 8000–10,000 blood trypomastigotes, 1 week later, there are about 30,000 parasites per posterior midgut. After blood-feeding the following instars, the numbers of trypanosomes increase in each successive nymphal instar, up to a mean of 600,000 parasites per fifth instar nymph.

In electron microscopy, the majority of flagellates are seen near the perimicrovillar membranes, and rarely in contact with the microvillar border of the cells of the intestinal wall (Kollien et al. 1998). Epimastigotes are proposed to attach to the midgut through different compounds, including cruzipain, heparin-binding molecules, cysteine peptidases, and glycoinositol phospholipids (e.g., Nogueira et al. 2007; Ennes-Vidal et al. 2011; Oliveira et al. 2012; Uehara et al. 2012; Cámara et al. 2019). In the binding studies that have been used to infer these molecular interactions, in vitro derived epimastigotes are often spun down at 10,000 g and then washed in protein-free buffer. However, centrifugation forces >400 g and the buffers used damage the surface of blood trypomastigotes (Hölscher et al. 2003). Since changing hormonal concentrations or feeding antibodies against perimicrovillar membranes strongly interferes with both the development of perimicrovillar membranes and the populations of *T. cruzi*, there is evidence that an interaction occurs between perimicrovillar membranes and *T. cruzi* (Gonzalez et al. 1999, 2006). Such interference is also demonstrated by supplementation of blood with ecdysone or azadirachtin, a neem tree compound that blocks the release of prothoracicotropic hormone from neurosecretory cells (Cortez et al. 2012). The interaction of epimastigotes with perimicrovillar membranes is complex, as there are continuous changes to the membranes, which develop after blood-feeding and are reduced during starvation (Billingsley and Downe 1983).

Triatomine starvation strongly affects the population density and the different stages of *T. cruzi* present in the posterior midgut. In an investigation of the *T. cruzi/T. infestans* combination from Chile, nymphs were infected with *T. cruzi* as first instars and then dissected as fifth instars (Kollien and Schaub 1998a). Fourth instar nymphs molt about 18 days after blood-feeding, then the final remnants of blood are transported to the posterior midgut. At 20 days after blood-feeding fourth instar nymphs, about 60,000 *T. cruzi* colonized the posterior midgut. After an additional 10 days of starvation, only 3000 parasites were present, and at 60 days or more of starvation, trypanosomes were no longer present (Kollien and Schaub 1998a). However, in other experiments on starvation capacity, all dead triatomines contained small populations of *T. cruzi* in the posterior midgut (Schaub and Lösch 1989).

Development of *T. cruzi* in the Rectum

The rectum contains only the remnants of blood meal digestion, but the highest density of trypanosomes develops there: in the *T. cruzi/T. infestans* combination originating from Chile, the *T. cruzi* population in the bug rectum was three times larger than the population in the posterior midgut (Schaub 1989a). One reason for the difference in parasite population density seems to be the possibility of optimal attachment, with about two-thirds of the rectal trypanosome population attached to the rectal cuticle. The epimastigotes possess a small hydrophobic region on the flagellum, which is able to bind to the wax layer that covers the whole rectal cuticle (Kleffmann et al. 1998; Schmidt et al. 1998). The Gp35/50 kDa protein is a trypanosome mucin, which seems to be important for parasite attachment (Cámara et al. 2019). The protein presumably covers the entire surface of the epimastigote cell body and not just the small hydrophobic binding region of the flagellum. In addition, not all strains of *T. cruzi* possess variants of the Gp35/50 kDa mucin that specifically determines adhesion.

Enlargements of the flagellum and hemidesmosome-like material beneath the parasite plasma membrane indicate the strength of attachment (Böker and Schaub 1984). The rectal pads are especially preferred. Studies of other insects have found a stronger absorption of water and amino acids at the rectal pads than in the other regions of the rectum (summarized by Schaub 1992). A “carpet” of up to four layers of epimastigotes covers the rectal cuticle, with those at the top being connected to the underlying rectal cuticle by a prolonged flagellum. In vitro, increases in the flagellar length are induced by glucose depletion (Tyler and Engman 2000, 2001). However, the importance of glucose in the rectum is questionable, and concentrations of monosaccharides present in the gut remain to be determined.

The attachment of *T. cruzi* to the rectal cuticle is important for metacylogenesis (Kleffmann et al. 1998), but metacyclogenisis can also occur in unattached epimastigotes. Comparing *T. cruzi* metacyclogenesis to the development of *Trypanosoma brucei* in the salivary glands of tsetse flies, in the latter, the metacyclic forms lose the ability to attach, either from the production of a new surface coat and/or a

shortening of the length of the free flagellum (Vickerman 1985). Metacyclic trypomastigotes of *T. cruzi* develop from both long and short epimastigotes, as well as spheromastigotes, as indicated by the translocation of the kinetoplast, and also by unequal cell division of the epimastigotes, resulting in one daughter epimastigote and one daughter metacyclic trypomastigote (Schaub and Lösch 1988; Schaub 1989a).

The proportions of intermediate stages differ during the course of an infection. About 10% of the trypanosome population in the rectum are intermediate stages of metacyclic trypomastigotes. The rectum contains the dark-brown remnants of blood meal digestion, rich in amino acids, and proteins. These conditions differ strongly from those used for in vitro metacyclogenesis assays (summarized by Gonçalves et al. 2018). Metacyclic trypomastigotes seem to occur only or mainly in the rectum. At 2 weeks p.i. with the strain *T. cruzi* Chile 5, already a quarter of the entire rectal population are trypomastigotes, which increases further to 50% from 10 weeks p.i. onwards. The strain *T. cruzi* Chile 7, also originating from the same location as the vector, reaches lower percentages of metacyclics (Schaub 1989a). Also in *R. prolixus* from Colombia, more trypanosomes overall, and more metacyclics in particular, develop in infections with the TcI than the TcII strain (Tamayo et al. 2018). In addition, after maintenance at 26, 28, and 30 °C, the number of metacyclic trypomastigotes is higher at the latter temperature than at other temperatures, and the period of time until their first appearance in the rectum decreased (Tamayo et al. 2018).

The molting process could also affect the rectal population, as it includes the withdrawal of the old rectal cuticle through the anus. Surprisingly, this seems to be of minor importance, having no strong effect on the trypanosome population size (Patterson and Miles 1973; Schaub 1989a). Fourth instar nymphs of *T. infestans* dissected 1 day after molting show no significant decrease in the rectal population size of trypanosomes (Schaub 1989a). In contrast, triatomine starvation strongly affects both the population density and the different stages of *T. cruzi* in the rectum. Initially, there are about 300,000 *T. cruzi* present in the rectum, but their population is reduced to about 1% of this number at 120 days after blood-feeding, although even then, all recta remain colonized (Kollien and Schaub 1998a). In scanning electron microscopy, residual *T. cruzi* populations always remain attached to the rectal pads, despite other intestinal regions being free of trypanosomes (Schaub and Böker 1986). The effect of starvation on the different stages of *T. cruzi* is very obvious (Kollien and Schaub 1998a). At 20 days after blood-feeding, 2% and 1% of the population are either spheromastigotes or drop-like forms, that is, intermediates between either sphero-, epi- or trypomastigotes, respectively.

Blood-feeding of the vector also has marked effects on established *T. cruzi* infections in the rectum (summarized by Kollien and Schaub 2000), with pH, osmolarity and ion concentrations in this intestinal region changing drastically (Kollien et al. 2001). The subpopulation of *T. cruzi* within the rectal lumen, and part of the subpopulation attached to the rectal wall, are flushed out by urine, reducing the entire trypanosome population by >50%. The deposited urine often contains pure populations of metacyclic trypomastigotes. At 40 days after blood-feeding of fourth instar

nymphs, as well as during blood-feeding of fifth instars, metacyclogenesis is induced in epimastigotes, but not in other precursor stages (Schaub and Lösch 1988). Within 4 h after blood-feeding, the proportion of slender intermediate stages increases significantly from <7% to 10%. During this period of time, metacyclogenesis is induced by hemolymph proteins of about 17 kDa that pass into the urine at the beginning of diuresis (Kleffmann 1999; Kollien and Schaub 2000). Since only metacyclic trypomastigotes survive in the blood of the mammalian host, this rapid induction of metacyclogenesis in the rectum increases the likelihood that the parasites excreted will be able to survive in the mammalian host, and, hence, increases the probability of successful transmission.

Blood-feeding after a starvation period of 60 days in fifth instar nymphs induces peculiar effects in the parasites. Before blood-feeding, 30% of the rectal population of trypanosomes are spheromastigotes (including intermediate forms), 20% are epimastigotes, and 50% are trypomastigotes (Kollien and Schaub 1998b). However, 1 day after blood-feeding the fifth instar nymphs, the proportion of these forms shifts to 2%, 70%, and 10%, respectively. In addition, at this time, there are about 10% "giant cells", that is, a multiple cell division stage. Up to 3 days after blood-feeding, the proportion of giant forms increases to on average 30–50% of the total parasite population, but between 5 and 10 days after feeding it disappears (Kollien and Schaub 1998b).

3.3 Effects of T. cruzi *on Triatomines*

The effect of *T. cruzi* infection on triatomines has been reviewed several times, and indicates that it either has only a weak pathogenicity or is subpathogenic, that is, pathologic effects only develop under adverse conditions, such as when the triatomines are simultaneously exposed to other stressors (Schaub 1989b, 1992, 2009; Garcia et al. 2010a; Schaub et al. 2011, 2016; Balczun et al. 2012a; Pausch et al. 2012; Guarneri and Lorenzo 2017). In the field, such adverse stressors include starvation and changes in temperature (Noireau and Dujardin 2001; Sarquis et al. 2010). In addition to environmental conditions, in laboratory investigations the blood source affects the development of triatomines (Guarneri et al. 2000; Heger et al. 2006; Rolandi and Schilman 2018). Therefore, this parameter should also be considered as a potential stressor capable of modulating the pathogenicity of *T. cruzi*. The distribution of infected and uninfected triatomines in the field has only recently been considered: Using species distribution models and comparing the ecological niches of seven Mexican species, the ecological niche used by *T. cruzi*-infected populations was often reduced in comparison to uninfected populations. The restricted niche amplitude of the infected triatomines is suggested to be caused by an effect of the trypanosomes on insect fitness (Villabolos et al. 2019).

In investigations of the interactions of trypanosomes with triatomines, the presence of mutualistic symbionts in the nymphs used is crucial, and needs to be carefully controlled, but is rarely considered. When beginning with experimental groups

of first instar nymphs, adult individuals or just feces can be added to the cohort to provide them with the opportunity to acquire symbionts from other triatomines. Alternatively, after microbiological identification of mutualistic symbionts of interest, the in vitro culture-derived bacteria can be given to experimental groups of nymphs after blood-feeding. When using the progeny of triatomines captured in the field, the transmission of any naturally-acquired *T. cruzi* infection must be avoided. The culture of rectal bacteria on agar plates, and supplying the progeny of field-captured triatomines with a mixture of rectal bacteria, seems to be optimal. In subsequent generations, adults or feces alone are sufficient for transmission of the triatomine microbiota (Schaub unpublished).

Effects of *T. cruzi* on the Development of Triatomines

The effect of *T. cruzi* on nymphal development differs according to the vector-parasite combination studied. In the Chilean combination described above, infected and uninfected first instars of *T. infestans*, which were raised either isolated or grouped together, molted after the same period of time (Schaub 1988b). In another vector-parasite combination, *T. cruzi* induced a retardation of the development of infected nymphs of *T. infestans*, especially the developmental time of individually maintained first instar nymphs, which increased fivefold (Reis dos Santos and Lacombe 1985). After blood-feeding on *T. cruzi*-infected mice, molting to the subsequent nymphal instar of *Mepraia spinolai* and *T. brasiliensis* is retarded relative to control triatomines blood-fed on uninfected mice (Botto-Mahan et al. 2008; Botto-Mahan 2009; Oliveira et al. 2010). In addition, nymphs of *M. spinolai* are significantly lighter than controls, and significantly more *T. cruzi*-infected fourth and fifth instar nymphs die in comparison to uninfected individuals (Botto-Mahan et al. 2008; Botto-Mahan 2009). In contrast, in the case of *T. brasiliensis*, nymphal developmental and mortality rates are unaffected when infected nymphs are blood-fed on uninfected mice in the following instars (Oliveira et al. 2010). Presumably, the concentration of essential unknown compounds and/or the general nutritive value of the blood of infected mice is reduced, causing negative effects on triatomines fed exclusively on them. However, in *R. prolixus* second instar nymphs fed once infectious or non-infectious blood via an artificial feeder and no blood afterwards, the molts of infected *R. prolixus* nymphs maintained at four different temperatures between 21 and 30 °C are strongly delayed by between 6 and 11 days (Elliot et al. 2015). Also, the mortality rates 90 days after infection are increased at both 24 °C and the normal maintenance temperature of 27 °C, but not at 21 and 30 °C (Elliot et al. 2015). Interestingly, trypanosome-associated mortality was highest over the range of temperatures in which *R. prolixus* is found in the wild (Heger et al. 2006).

No effects of trypanosome infection on nymphal development occur in *P. megistus, T. brasiliensis* and *T. infestans* (Juarez 1970; Schaub 1988c; Lima et al. 1992; Oliveira et al. 2010). After infecting *T. infestans* and *T. brasiliensis* by feeding them on mice infected with a *T. cruzi* strain isolated from triatomines from the same

location, nymphs fed on uninfected mice or chickens have normal developmental times (Juarez 1970; Schaub 1988c). In the Chilean vector-parasite combination, the mortality rates of infected and uninfected nymphs of *T. infestans* fed on chickens are identical (Schaub 1988c). The loss of nutrients from trypanosome-infected blood can be compensated by either increasing the volume of blood ingested and/or the number of blood-feeds given to triatomines (Juarez 1970). Nevertheless, it is worth mentioning that in the field, blood meals are often difficult for triatomines to obtain, and starvation conditions are not uncommon (Noireau and Dujardin 2001; Sarquis et al. 2010).

In the Chilean *T. infestans-T. cruzi* combination, parasite effects on the starvation capacity of the vector are evident. After trypanosome infection of first instar nymphs, followed by either one, two or three additional uninfected blood meals to the subsequent nymphal instars, the mean period of starvation resistance of the resulting third, fourth and fifth instar nymphs is reduced by 3%, 14%, and 17%, respectively, relative to uninfected triatomines, with the two last differences being statistically significant (Schaub and Lösch 1989). Since more infected than uninfected nymphs contain the brown hemoglobin remnants of the blood meal in their posterior midguts, *T. cruzi* and its vector do not seem to compete with each other for the hemoglobin, but for essential metabolites whose depletion results in death. An accumulation of toxic products by *T. cruzi* seems to be unlikely, since many trypanosomes die in starved triatomines (Schaub and Böker 1986; Kollien and Schaub 1998a). Also in *Triatoma* (*Meccus*) *pallidipennis* after an infection of fifth instar nymphs with *T. cruzi* strains from the same location, the period of time until death in the following starvation is shorter in infected than uninfected nymphs (González-Rete et al. 2019). In *M. spinolai* individuals collected from the field, and blood-fed within 2 weeks after collection, and then again 40 days later, and maintained separately until their death, infection with *T. cruzi* does not affect the starvation capacity of either third and fourth instar nymphs or adults (Mc Cabe et al. 2019).

Studies on the effect of trypanosome infection on the longevity and fecundity of triatomine adults also show contradictory results. No effects of *T. cruzi* infection on either the longevity or fecundity of *T. brasiliensis*, *T. dimidiata* and *T. infestans* were observed (e.g., the mean lifespan of both adult males and females, as well as the hatching rate of eggs, the period of time before oviposition, the number of ovipositions, and both the total number of eggs laid and number of fertile eggs were not different compared to uninfected controls, if the infected adults were fed on uninfected blood) (Zeledón et al. 1970; Schaub et al. 1985; Oliveira et al. 2010). However, in *T. infestans* infected with *T. cruzi*, both the egg-laying rate during the first weeks of oviposition and the hatching rate may be reduced slightly (Schaub et al. 1985).

In *M. spinolai* fed on *T. cruzi*-infected mice, the weight of the gonads was reduced in comparison to control triatomine females fed on uninfected mice, as a consequence of overall decreased body size (Botto-Mahan et al. 2008). The effects of *T. cruzi* on this triatomine species are sex-dependent (Botto-Mahan et al. 2017). As the triatomines in these experiments were only fed on infected mice, the observed effects might be related to a lower concentration of essential unknown compounds

and/or a lower general nutritive value of the blood of infected mice. After infection of second instar nymphs of *R. prolixus* using a mixture of epimastigotes and citrated rabbit blood, which were then maintained at either 25 °C or 30 °C, and fed uninfected blood in the following instars, the period of time before the first egg laying was similar to that of uninfected controls (Fellet et al. 2014). The capacity to convert ingested blood into eggs was increased in triatomines kept at 25 °C in the second reproductive cycle of blood-feeding, while fecundity was decreased in the first reproductive cycle of adults raised at 30 °C. In the latter, significantly fewer nymphs hatched from eggs laid in the third reproductive cycle (Fellet et al. 2014). After blood-feeding first instar nymphs of *P. megistus* on infected mice, and blood-feeding the following instars on defibrinated sheep blood, the number of eggs produced by *T. cruzi*-infected couples, as well as the number of fertile eggs and hatched nymphs were significantly reduced (Lima et al. 1992). These effects and a reduced survival also occur after infection of fifth instar nymphs of *T.* (*M.*) *pallidipennis* on mice and a subsequent feeding on uninfected mice (Cordero-Montoya et al. 2019). In a study of Colombian *R. prolixus* fifth instar nymphs experimentally infected with five different Colombian TcI strains, longevity post-infection varied by *T. cruzi* strain; some *T. cruzi* strains had no effect compared to uninfected triatomines, while others significantly reduced survival and retarded development. The nymphs were fed on live chickens, aside from the infective blood meal, which consisted of *T. cruzi* epimastigotes in defibrinated, decomplemented human blood administered through a membrane feeder (Peterson et al. 2015). Using the same methodology, but infecting the bugs with either single infections of *T. cruzi* (TcI) or *T. rangeli* or co-infecting bugs with *T. cruzi* and *T. rangeli*, the survival and reproduction of co-infected adults was better than that of both uninfected and only singly-infected adults (Peterson et al. 2016). The life-expectancies of *T. cruzi*-infected *Triatoma* and *R. prolixus* are also reduced (Carcavallo 1970; Neves and Peres 1975). *Rhodnius prolixus* blood-fed as first instar nymphs on *T. cruzi*-infected guinea pigs, and then blood-fed on uninfected guinea pigs in the following instars, showed a reduction in longevity of 6 months when compared with uninfected but otherwise similarly blood-fed control triatomines (Neves and Peres 1975). A reduction in longevity of 8 months was observed when the triatomines were always fed on *T. cruzi*-infected guinea pigs.

In summary, with regard to trypanosome interactions with adult triatomines, the effect observed in some investigations might be due to a decrease in the nutritional quality of the blood from infected vertebrate hosts. However, some strains of *T. cruzi* seem to affect the vector even under optimal conditions.

Effects on Behavior

Triatomines show a wide range of different behaviors (summarized by Lazzari et al. 2013; Barrozo et al. 2017), many of which are connected to hematophagy. So far, the majority of these behaviors has not been compared between *T. cruzi*-infected and uninfected triatomines.

The most studied behaviors are those related to triatomine orientation to the host, blood-feeding and subsequent defecation. After infection of second instar nymphs of *T. longipennis* and *T.* (*M.*) *pallidipennis* by feeding them on mice infected with Mexican strains of *T. cruzi,* only nymphs in the third instar but not in the fourth and fifth instar react more rapidly to human odor than uninfected nymphs (Ramírez-González et al. 2019). After infection in the first instar, feedings on uninfected mice in the following instars and a starvation for 7 weeks after molting, *T. cruzi*-infected fifth instar nymphs of *M. spinolai* orient themselves to their vertebrate host twice as fast as uninfected nymphs (Botto-Mahan et al. 2006). In these nymphs, the number of bites divided by the feeding time is nearly doubled by triatomine infection with *T. cruzi*, while infected nymphs also defecate sooner after the blood meal. The sooner defecation is also evident in fifth instar *T. infestans* nymphs that were infected with *T. cruzi* as third instars through feeding on mice infected with the *T. cruzi* strain Tulahuen (TcVI). The nymphs were maintained by feeding on live chickens in addition to one blood meal from live pigeons at 15–20 days after the imaginal molt (Pereyra et al. 2020). *T. cruzi*-infected nymphs also defecate within 10 min after ingestion of uninfected blood, and they deposit larger quantities of feces/urine than do uninfected nymphs. In contrast, *R. prolixus* nymphs that are naturally infected with *T. cruzi* feed less frequently than uninfected nymphs (D'Alessandro and Mandel 1969). However, after experimental infection, and 2–3 weeks after molting, infected and uninfected nymphs and adults of this species possess identical times for feeding on live hosts, blood meal weights, number of probes, and time to first defecation (Takano-Lee and Edman 2002). These discrepancies in feeding and defecation behavior could be due to differences in triatomine species or parasite strains, duration of *T. cruzi* infections and/or the starvation periods before recording the behaviors. If *T. cruzi* and the vector compete for components in the blood, then infected nymphs may possess a more advanced state of starvation in comparison to uninfected nymphs. Interestingly, in a field study, in which humans were used as hosts to attract *M. spinolai*, more *T. cruzi*-infected bugs were captured within the first hour of exposition. The infected triatomines presented a decreased standardized body mass index than unifected ones, reinforcing the idea that the behavioral changes observed can be related to a reduction in the nutritional resources promoted by the infection (Estay-Olea et al. 2020).

Differences in the availability of nutritional supply can also explain the following behavioral variations. The locomotion of *R. prolixus* is decreased after infection of second instar nymphs with *T. cruzi* epimastigotes, with nymphs feeding on uninfected mice in the subsequent instars. In these bugs, the nocturnal locomotion events of fifth instars are reduced by 20% as compared to uninfected individuals, although phototaxis remains unchanged (Marliére et al. 2015). The dispersal capabilities of *T. dimidiata* do not differ between *T. cruzi*-infected and uninfected males, but are increased in infected females compared to uninfected females (Ramirez-Sierra et al. 2010). These differences between genders cannot be explained by wing sizes, because *T. cruzi*-infected males and females collected in the field have larger wings than uninfected individuals (Nouvellet et al. 2011). *T. cruzi*-infected females of *R. pallescens* show an increased flight speed in comparison to infected males at 30 s

and 2 min in experiments performed on a tethered flight mill (Castro et al. 2014). Since the initiation of flight is also increased during starvation (Schofield 1979), *T. cruzi* infections might have a greater effect on the nutritional status of infected females than on infected males, increasing their ability to disperse. *T. cruzi* infection also induces higher levels of negative geotaxis and higher aggregation levels in both female and male adults of *T. infestans*, as recently shown in laboratory-controlled experiments (Depickère et al. 2019).

Effects on Immunity

Like other insects, triatomines possess an immune system comprised of humoral and cellular components (Müller et al. 2008). So far, immune cells have only been found in the hemolymph, and not in any section of the intestine within which *T. cruzi* develops. Triatomine humoral immunity is mediated by many different antimicrobial peptides (e.g., lysozymes, defensins), several unidentified bacteriolytic compounds, and antimicrobial molecules (e.g., nitric oxide). Antibacterial activity is visible in zymograms including the bacterium *Micrococcus luteus* (syn. *M. lysodeikticus*, Waniek et al. 2009). At 1 day after blood-feeding of fifth instar nymphs of *T. infestans*, the antibacterial activities apparent on zymograms are about 50% lower than in unfed nymphs in both regions of the midgut, and increase up to 40 days after blood-feeding to the level of unfed nymphs (Meiser 2009). In addition, the antibacterial activities are much higher in the anterior midgut than in the posterior midgut, also in the Mexican *T.* (*M.*) *pallidipennis* system at 15 days after feeding for the prophenoloxidase activities (González-Rete et al. 2019). Using whole mount in situ hybridization, the genes encoding defensin and lysozyme are highly expressed in the anterior midgut, but at only very low levels in the posterior midgut (Kollien et al. 2003; Araújo et al. 2006). Some of these antimicrobial peptides are encoded by different genes, as in the case of *T.* (M.) *pallidipennis,* where 12 different genes code for three mature peptides with the typical folding of a functional defensin (Díaz-Garrido et al. 2018).

T. cruzi infections in the triatomine intestine also affect the immune response in the hemolymph and other organs (summarized by Schaub 2009). In *R. prolixus*, immune responses are induced by infection with *T. cruzi* blood trypomastigotes (Ursic-Bedoya et al. 2008). Using the whole intestine, at 7 and 14 days p.i. the expression of the gene of the most intestinally active lysozyme, RpLys-A, is increased >20-fold, while that of the lysozyme RpLys-B, the gene for which is primarily expressed in the fat body, is not altered. In the posterior midgut of *T. brasiliensis*, the expression of the gene encoding the defensin Def1 is 9.6-fold higher at 20 days p.i. with epimastigotes of the *T. cruzi* strain TBRA/BR/1999/JCA3 (TcII) compared to uninfected nymphs (Waniek et al. 2011). Concentrations of the antimicrobial molecule nitric oxide cannot be determined directly, but concentrations of its metabolite nitrite increase in the posterior midgut after an infection with blood trypomastigotes, and the expression of the gene encoding nitric oxide synthase increases in the anterior midgut when the development of *T. cruzi* is confined to this

region (Whitten et al. 2007). In comparison to uninfected blood-fed controls, there is a statistically significant increase in the levels of nitrite in both midgut regions at 1 and 2 days, as well as 2 weeks, after an infection with blood trypomastigotes of the *T. cruzi* strain Chile 5, although no parasites are present in the anterior midgut at 2 weeks p.i.. The nitrite levels in the rectum are also significantly higher at both phases of the infection, even before the parasites develop in the rectum (Whitten et al. 2007). In the anterior midgut of *T.* (*M.) pallidipennis* infected with blood trypomastigotes, the activities of prophenoloxidases are significantly higher in infected fifth instar nymphs at 28 days p.i., while those of phenoloxidases are significantly lower at 9 days p.i. (Favila-Ruiz et al. 2018). In this system, the increase in prophenoloxidase activity is also evident at 15 days p.i. and at 20 °C, but not at 30 °C and 34 °C (González-Rete et al. 2019) In the anterior midgut of *T. infestans* at 24 h p.i. with trypomastigotes of the *T. cruzi* strain Y (TcII), expression of the gene that encodes a cysteine protease inhibitor is significantly upregulated in comparison to uninfected adults (Buarque et al. 2011). In addition, the expression of genes encoding lysozyme, cathepsin D, a nitrophorin-like protein and a putative 14 kDa protein are all significantly upregulated, while the gene encoding thioredoxin reductase is downregulated. Expression for genes encoding infestin, lipocalins, and defensins are unchanged (Buarque et al. 2013).

Pathogen-associated molecular patterns inducing triatomine immune reactions are presumably present in the surface coat of the trypanosomes. The trypanosome surface coat is highly organized, containing lipid-driven domains with different protein composition (e.g., Mucci et al. 2017). Given that experimental shedding of the surface coat of blood trypomastigotes can be induced by strong centrifugation forces and incubation in protein-free buffer (Hölscher et al. 2003), as well as the effect of shearing forces created during forced passage through a fine (i.e., high gauge) syringe needle, it is possible to feed either the separated surface coat or the resulting "naked" trypomastigotes to triatomines (Pausch 2012). Up to 5 days after feeding fifth instar nymphs of *T. infestans* with complement-inactivated rat blood mixed with either separated surface coats or "naked" trypomastigotes or epimastigotes of the *T. cruzi* strain Chile 5, or blood-feeding them on anesthetized and normally *T. cruzi*-infected rats, antibacterial activity is significantly increased in the posterior midgut of nymphs that ingested either separated surface coats or intact blood-stage trypomastigotes, but not "naked" trypomastigotes or in vitro culture-derived epimastigotes (Pausch 2012).

Interaction of *T. cruzi* and the Microbiota of Triatomines

The effects of the triatomine microbiota on *T. cruzi*, and the effects of *T. cruzi* on the triatomine microbiota (including interactions with mutualistic symbionts of triatomines), can be conceptually separated. Effects of microbiota on *T. cruzi* are especially evident for *S. marcescens*, a bacterium found in triatomines collected from the field (e.g., Azambuja et al. 2004; Castro et al. 2007; da Mota et al. 2012, 2019; Gumiel et al. 2015; Oliveira et al. 2018). This bacterium lyses epimastigotes of the

T. cruzi Y strain through D-mannose recognizing fimbriae of the bacteria adhering to the trypanosome surface (Azambuja et al. 2004; Castro et al. 2007). A genetically-transformed mutualistic symbiont, *Rhodococcus rhodnii*, of *R. prolixus* also inhibits the development of *T. cruzi* by producing a lepidopteran antibacterial peptide or dsRNA and a corynebacterial symbiont of *T. infestans* by producing an antibody fragment (Beard et al. 2002; Durvasula et al. 2008; Taracena et al. 2015).

Indirect interactions between *T. cruzi* and triatomine microbiota can occur through competition for resources, or the induction of the humoral immune responses of the triatomine by *T. cruzi* or the bacteria (Azambuja et al. 2005a; Garcia et al. 2010b). Such interactions are evident immediately after the ingestion of trypanosome-infected blood. After feeding *T. infestans* nymphs on mice infected with the *T. cruzi* CL strain, the interaction between humoral immunity and the comparative population sizes of the trypanosomes and the bacterial microbiota are striking: after knockdown of an undescribed antimicrobial protein named TiAP and infection with *T. cruzi,* at 3 h p.i., the number of trypanosomes in the anterior midgut is significantly lower and the number of bacteria 600-fold higher than in control *T. infestans* in which the antimicrobial protein was not silenced (Buarque et al. 2016). Additionally, a protease Kazal-type inhibitor—rRpTI—was characterized in *R. prolixus* (Soares et al. 2015), and the single domain inhibitor was suggested to function as an antibacterial protein (Meiser et al. 2010). Usually, these molecules inhibit the clotting of ingested blood in the anterior midgut (Meiser et al. 2010). In different triatomines, the open reading frame encodes a putative precursor protein containing multiple Kazal-like domains, which are suggested to be processed post-translationally into individual inhibitors (Lovato et al. 2006). In the case of rRpTI, 3 h p.i. of *R. prolixus* with blood trypomastigotes, it is significantly upregulated in the anterior midgut (Soares et al. 2015). After knockdown of the respective transcript, at 3 h p.i. with the *T. cruzi* strain CL the number of trypanosomes is lower, and the bacterial load higher, compared to the control group triatomines. Therefore, blood trypomastigotes seem to induce a short-term upregulation of antimicrobial proteins that modulate (i.e., suppress) bacterial populations. Similar phenomena also seem to occur with fungi: after experimental xenodiagnosis, *P. megistus* infected with *T. cruzi* possess much smaller fungal populations (Moraes et al. 2001b).

There are also longer-term effects of trypanosome induction of humoral immunity on the triatomine microbiota. Eight to 13 days after feeding *R. prolixus* defibrinated rabbit blood mixed with 1×10^7 epimastigotes of the *T. cruzi* strain Dm28c per ml, and antibiotics to act against the triatomine microbiota, the entire triatomine intestine contains 10-times more trypanosomes than those of control triatomines (Castro et al. 2012a). At 7 days p.i., the mRNA levels of defensin C and prolixicin, as well as the activity of phenoloxidase and antibacterial activity are all increased, while nitrite and nitrate production are reduced and the populations of bacteria are decreased (Castro et al. 2012a; Vieira et al. 2016). In the same vector-parasite combination, the opposite effects occur 8 or 9 days after a feeding on rabbit blood mixed with physalin B, a plant-derived immune modulator of *R. prolixus*. In this case, using the entire intestine, antibacterial activity is decreased, nitrite and nitrate levels as well as the numbers of bacteria are increased, and the development of *T. cruzi* is

strongly or totally inhibited (Castro et al. 2012b). An increase in the species diversity of the microbiota of 2–4 laboratory-bred *P. megistus*, *T. brasiliensis* and *Triatoma sherlocki* individuals is evident 10 days after blood-feeding a mixture of decomplemented rabbit blood and *T. cruzi* epimastigotes of a TcI strain (Díaz et al. 2016).

In *T. brasiliensis* (nymphs and adults) and *T. pseudomaculata* (mainly adults) captured from the field, the presence of trypanosomatids does not affect the bacterial species composition of the microbiota (Gumiel et al. 2015). In field-caught *T. cruzi*-infected *T. infestans*, the relative abundance of 10 specific bacterial taxa are significantly different from those present in the uninfected *T. infestans* collected in the same locations (Waltmann et al. 2019). The presence of *T. cruzi* in field-collected *T. dimidiata* is associated with an increase in the diversity of the bacterial microbiota; while 508 putative bacterial species are present in uninfected adults, 1006 are present in *T. cruzi*-infected ones (Orantes et al. 2018). In *T. cruzi*-infected *T. protracta* adults captured in the field, the diversity of the microbiome is significantly higher than in uninfected adults (Rodríguez-Ruano et al. 2018). An increase in the diversity of the bacterial microbiota is also evident in fifth instar nymphs of *T. infestans* with *T. cruzi* infections resulting from experimental infection of third instar nymphs with a mixture of blood trypomastigotes and different microorganisms in the blood meal. High numbers of fungi and bacteria develop only in *T. cruzi*-infected fifth instar nymphs, but not in uninfected controls, suggesting immune suppression in the intestine (Eichler 1998). Although it is not possible for triatomines captured from the field to know how long they have been infected with trypanosomes, those *T. cruzi*-infected adult triatomines which possess an increase in the diversity of the bacterial microbiota seem to be long-term infected.

Finally, the fore-going interactions with the bacterial microbiota must be considered separately from the interactions with mutualistic symbionts. After infection of axenic first instar nymphs of *R. prolixus* and *T. infestans* with their respective mutualistic symbionts, *R. rhodnii* and *Rhodococcus triatomae*, and subsequent infection with blood trypomastigotes of *T. cruzi* in the fifth instar, the numbers of symbionts in the different regions of the intestine are not affected by the presence of trypanosomes during the first 10 days p.i. (Eichler and Schaub 2002). At six and 24 h p.i. of axenic first instar nymphs of *R. prolixus* infected with cell-culture-derived trypomastigotes—which resemble blood trypomastigotes—the number of trypanosomes is similar in both groups. In other words, the presence of the mutualistic symbiont does not interfere with the development of *T. cruzi* and vice versa (Dias et al. 2015).

4 Interactions of Triatomines with *T. rangeli*

4.1 The Parasite

Trypanosoma rangeli is a hemoflagellate parasite of triatomines and mammals in Central and South America, including humans and their associated domestic animals (Grisard et al. 1999). In mammalian hosts, the development of *T. rangeli* is poorly understood, since the multiplicative forms are so far undescribed. In triatomines, *T. rangeli* develops in the intestinal tract, hemolymph, and salivary glands. According to kDNA and nuclear DNA analysis, *T. rangeli* has very high levels of genetic variability (Steindel et al. 1994; Maia da Silva et al. 2007). kDNA minicircle sequences can present molecules with one (KP1), two (KP2) and four (KP3) conserved regions (Vallejo et al. 1994). Based on this, two major lineages of *T. rangeli* have been characterized: strains that give KP1, KP2, and KP3 minicircle PCR amplification products are termed KP1(+), while those that give amplification products derived only from KP2 and KP3 minicircles are termed as KP1(−) (Vallejo et al. 2002, 2003). Based on small subunit rDNA, ITS-1 and the spliced-leader intergenic region sequences, lineages denoted as A, B, C, D, and E have also been described (Maia da Silva et al. 2004, 2009). More recently, single-nucleotide polymorphism and microsatellite analyses suggest that there are three *T. rangeli* groups: Amazonian, KP1(−) and KP1(+) (Sincero et al. 2015).

Trypanosoma rangeli can infect different genera of triatomines, and both natural and experimental infections have been reported in species of *Triatoma* and *Panstrongylus* (summarized by Guarneri and Lorenzo 2017). However, hemolymph infection in *Triatoma* and *Panstrongylus* species is uncommon, and invasion of salivary glands is even more rare (D'Alessandro 1972; Tovar et al. 1989). In contrast, the genus *Rhodnius* is usually susceptible to infection by *T. rangeli*, and transmission by salivary inoculation has been demonstrated in 13 of the 20 nominal *Rhodnius* species using either naturally- and/or experimentally-infected insects (*R. brethesi*, *R. colombiensis*, *R. dalessandroi*, *R. domesticus*, *R. ecuadoriensis*, *R. montenegrensis*, *R. neglectus*, *R. nasutus*, *R. neivai*, *R. pallescens, R. pictipes*, *R. prolixus,* and *R. robustus*) (Vallejo et al. 2015). Studies prior to the genetic characterization of the *T. rangeli* groups had already identified differences in the development of this trypanosome between *Rhodnius* species (Tobie 1961; Zeledón and Blanco 1965; D'Alessandro 1972; Machado et al. 2001). KP1(−) *T. rangeli* strains isolated from different triatomine species in the *R. pictipes* lineage (comprised of Andean and Amazonian species, such as *R. pallescens*, *R. pictipes, R. colombiensis,* and *R. ecuadoriensis*) are genetically divergent from the KP1(+) *T. rangeli* strains isolated from the *R. robustus* lineage (comprised of the Amazonian species *R. robustus* sensu lato and *R. prolixus*, as well as species that occur in other ecoregions, such as *R. nasutus*, *R. neglectus,* and *R. domesticus*). The genetic divergence indicates possible differences in the susceptibility of vectors to different genotypes of *T. rangeli* (Urrea et al. 2005; Vallejo et al. 2007). Moreover, the invasion of salivary glands and transmission by bite of specific *T. rangeli* strains is mostly limited to local vector species,

suggesting a strong co-evolutionary association between *T. rangeli* isolates and their sympatric vectors (Guhl and Vallejo 2003; Vallejo et al. 2003; Maia da Silva et al. 2007; Urrea et al. 2005, 2011). A trypanolytic protein isolated from *R. pallescens*, *R. colombiensis* and *R. ecuadoriensis*, that selectively lyses KP1(−) but not the KP1(+) strains, was partially characterized from the *R. prolixus* hemolymph and may explain the resistance of triatomines to allopatric strains of *T. rangeli* (Sánchez et al. 2005; Pulido et al. 2008).

4.2 Development of T. rangeli *in the Vector and Effects of the Vector on* T. rangeli

Development of *T. rangeli* in the Midgut

The intestinal forms of *T. rangeli* were first described by Tejera (1920) and Rey Matiz (1941). The parasite is ingested when a triatomine feeds on an infected mammal, and can persist in the insect vector throughout its lifespan. The number of parasites initially ingested is believed to be low, as the parasitemias, at least in experimentally-infected mammals, are usually low or non-apparent (Molyneux 1973; Urdaneta-Morales and Tejero 1985; Zuñiga et al. 1997). In spite of low infective doses, *T. rangeli* transmission rates from the vertebrate host to the triatomine vectors are very efficient, with rates of ~80%, even in mice with long-term infections (Ferreira et al. 2015). *Trypanosoma rangeli* can also be transmitted from triatomine to triatomine through hemolymphagy (i.e., when triatomines ingest hemolymph from feeding on other triatomines, Añez 1982), or when trypanosome-infected and uninfected individuals share the same uninfected vertebrate host during blood-feeding (Cuba Cuba 1972; Ferreira et al. 2015). In the latter instance, the triatomine to triatomine transmission can occur even if the vertebrate host species is not susceptible to infection with the parasite, as is the case with birds (Ferreira et al. 2015).

A few hours after triatomine ingests blood infected with *T. rangeli*, blood trypomastigotes start to be replaced by rounded and intermediate forms in the anterior midgut. Epimastigotes, the multiplicative form of the parasite, appear from the first day onwards (Añez 1983a; Ferreira et al. 2018), and all parasite forms multiply by binary division. *T. rangeli* colonizes and multiplies in all regions of the triatomine intestinal tract, but preferentially in the anterior and posterior midgut. Parasite growth is affected by environmental temperature during the beginning of the infection. Afterward, the parasite burden is mostly regulated by nutritional resources, as infection can be eliminated from insects by prolonged starvation (Ferreira et al. 2018). While in the midgut, the parasites stay free in the lumen (Hecker et al. 1990).

Development in the Hemolymph

In contrast to development in the gut, crossing the wall of the intestinal tract and colonization of the hemolymph are not trivial for *T. rangeli*. Invasion of the hemolymph, when it occurs, seems to be by chance, and independent of the age and sex of the triatomine, as well as parasite load. As observed in other hematophagous arthropods, which are commonly infected by pathogens that colonize the salivary glands (Dyer et al. 2013; Franz et al. 2015), the intestinal epithelium of triatomines possibly acts as a mechanical/physiological barrier to *T. rangeli*. For example, the exposure of *R. prolixus* to gamma radiation caused alterations in the ultrastructural organization of the perimicrovillar membranes and microvilli of the triatomine midgut epithelium, facilitating the invasion of the hemolymph by *T. rangeli* (Gomes et al. 2002).

In both naturally- and experimentally-infected triatomines, crossing rates range from 2% to 50% (Groot 1954; Tobie 1965, 1970; Marinkelle 1968; Cuba Cuba 1974; Añez et al. 1987; Hecker et al. 1990; Ferreira et al. 2010), although 100% rate of hemolymph invasion has been reported in an experimental infection that used a recently isolated *T. rangeli* strain (Marquez et al. 2006). These characteristics make it difficult to study the process of trypanosome passage across the intestinal barrier. Only two studies have described the passage of *T. rangeli* through the intestinal epithelium using transmission and scanning electron microcopy. Using in vitro experiments, Oliveira and Souza (2001) showed that *T. rangeli* adhered to epithelial cells through either their posterior end or flagellum. Several trypanosomes can penetrate the same epithelial cell, which may be damaged during this process. According to in vivo experiments conducted as part of the same study, prior to epithelial cell penetration, the trypanosomes adhered to the extracellular membrane layers. Hecker et al. (1990) showed that 2–5% of *R. prolixus* nymphs orally-infected with one of two different *T. rangeli* strains had parasites in the hemolymph. In additional transmission electronic microscopy studies of triatomine individuals with known trypanosome infection, it was possible to observe parasites penetrating the midgut epithelium through an intracellular route but enclosed by a vacuole. The vacuoles contained either one or several parasites, and after crossing the basal lamina the parasites left the vacuole. The presence of host membranes around the vacuole, and the orientation of the parasites in various directions, suggested that the parasites are phagocytized and escape from the vacuole through active rotation and movements (Hecker et al. 1990).

Just after crossing the intestinal tract, only short epimastigotes are seen in the hemolymph. After that, intermediate and long epimastigotes are present in the hemolymph, and all of them are able to multiply (Añez 1983b). Consequently, the number of trypanosomes increases during infection of the hemolymph (Takle 1988; Oliveira and Souza 2003; Ferreira et al. 2010), and parasites inside hemocytes are commonly observed. In addition, hemocytes—mostly plasmatocytes—aggregate in close association with the trypanosomes (Takle 1988). Some authors report multiplication of trypanosomes inside these host cells, which break up and release rounded parasite forms (Pifano and Mayer 1949; Coutinho and Nussenzweig 1952;

Tobie 1970; Watkins 1971a; Añez 1983b). However, Oliveira and Souza (2003) did not find intracellular multiplication in plasmatocytes. Rather, they found only rounded parasites showing various degrees of degeneration, presumably indicative of parasite lysis. The differences reported in these studies are possibly due to the specific characteristics of the different *T. rangeli* strains used, for example, KP1(−) (+) strain/vector species pairings.

The presence of *T. rangeli* also produces physiological changes in the hemolymph, such as an alteration in the amount of free amino acids (Ormerod 1967), an increase in the volume of hemolymph (Grewal 1957; Watkins 1971b; Ferreira et al. 2010), and an increase in the amount of lipids (Ferreira et al. 2010). Lipids, such as diacylglycerol and phospholipids, are transported in the hemolymph by the lipid carrier lipoprotein lipophorin (Atella et al. 1995). Interestingly, *T. rangeli* internalizes neutral lipids and phospholipids from the hemolymph of *R. prolixus* together with their associated lipophorin through a specific receptor on the parasite surface (Folly et al. 2003). Therefore, either an imbalance in the levels of lipophorin in the hemolymph due to its incorporation by the trypanosomes, or changes in lipid metabolism, may result in increasing amounts of fat being available in the hemolymph.

Development in the Salivary Glands

In order to complete their developmental cycle, *T. rangeli* epimastigotes that multiply in the hemolymph must invade the salivary glands, and transform into metacyclic forms that will be infective to the mammalian host. In *Rhodnius* species infected by sympatric *T. rangeli* strains, once the hemolymph is invaded, the salivary glands usually also become infected. The colonization of the salivary glands begins 7 days after the trypanosomes reach the hemolymph (Ellis et al. 1980; Kitajima et al. 1998; Paim et al. 2013). At approximately 25 days after hemolymph invasion, the rates of salivary gland infection are close to 100% (Paim et al. 2013). However, environmental temperature can alter the salivary gland invasion rate in a non-linear manner; salivary gland infection rates in *R. prolixus* fifth instar nymphs kept at either low (21 °C) or high (30 °C) temperatures decreased by 47.4% and 61.5%, respectively (Rodrigues et al. 2016). In another study, *R. prolixus* inoculated with a *T. rangeli* KP1(+) strain recently isolated from domestic *R. prolixus* collected in the field had a higher number of parasites inside the salivary glands when kept at 25 °C in comparison to insects kept at 30, 35, and 40 °C (Hinestroza et al. 2016).

One pair of salivary glands is present in *R. prolixus*, each of which consists of a single reddish principal lobe and a single translucent accessory gland (Meirelles et al. 2003). The principal lobe is composed by a single epithelial layer of glandular binucleated cells surrounding a single large central luminal chamber where the secretory material is stored. The epithelial cell layer is enclosed by a double layer of smooth muscle cells that are in turn, surrounded by a basal lamina (Baptist 1941; Nussenzveig et al. 1995; Meirelles et al. 2005). Invasion of the salivary glands is started by long epimastigotes that can be seen either isolated or in clusters in

apparent contact with the basal lamina, flagellum foremost, but without cellular structures indicative of attachment (Ellis et al. 1980; Kitajima et al. 1998; Basseri et al. 2002; Meirelles et al. 2005). Entry into the salivary glands is apparently started after molecular recognition and adhesion, and possibly involves specific interactions between carbohydrates and lectins present on the surface of both the epimastigotes and the salivary glands (Basseri et al. 2002). Ectophosphatases present in both the trypanosomes and the salivary glands themselves also seem to play a role in parasite invasion. Long epimastigotes are able to modulate the endogenous ectophosphatase activity of salivary gland cells via a D-galactose and specific lectin-receptor interaction (Gomes et al. 2008). Additionally, the dephosphorylation of tyrosine residues by tyrosine phosphatases of the trypanosomes seems to be part of the invasion process, as parasite strains with a low activity of these enzymes have decreased salivary gland invasion efficiency (Dos-Santos et al. 2012). After the penetration of the basal lamina, the trypanosomes enclosed in vacuoles pass through the epithelial cells, from where they escape once they reach the lumen of the salivary gland (Ellis et al. 1980; Hecker et al. 1990; Kitajima et al. 1998; Meirelles et al. 2005). The trypanosomes remain extracellular and free within the saliva itself, where they differentiate into infective metacyclic forms. Twenty-five days after initial infection of the triatomine hemolymph, *T. rangeli* metacyclic stages represent 95% of the total number of trypanosomes present in the salivary glands. At this time, an average of 100,000 parasites colonizes each salivary gland (Paim et al. 2013). The trypanosomes apparently use the proteins stocked in the lumen of the salivary glands as an energy source until they are transmitted to the mammalian host (Paim et al. 2013).

4.3 Effects of T. rangeli *on Triatomines*

As mentioned, *T. rangeli* is a parasite that colonizes both the gut and the hemocoelomic cavity of triatomines. This trypanosome multiplies in all regions of the intestinal tract, as well as in the hemolymph, besides maintaining a large population of metacyclic forms in the salivary glands. In experimental infections, using the *R. prolixus*- *T. rangeli* Choachi strain combination, initiated either orally or by intracoelomic injection, it is not unusual to find insects with impressive numbers of parasites found in their tissues, similar to the artificially high parasite densities typically only observed in in vitro cultures. It is, therefore, not surprising that such massive infections would be pathogenic to triatomines. Such pathogenic effects of *T. rangeli* to its invertebrate hosts have been extensively reported. It is worth mentioning that most pathogenic effects have been reported in studies using *R. prolixus*, and thus may not be generalizable to all other triatomine species (Peterson and Graham 2016).

Effects of *T. rangeli* on the Development of Triatomines

The most frequently observed pathogenic effect of *T. rangeli* infection is an increase in triatomine mortality rates, mostly during ecdysis, but also often in engorged individuals (Grewal 1957; Tobie 1965; Watkins 1971a, b; Añez 1984; Cuba Cuba 1998). Molts are often delayed or fail to occur (Grewal 1957; Tobie 1965; Cuba Cuba 1998), and when molting does occur, newly molted individuals frequently exhibit deformities (Watkins 1971a; Cuba Cuba 1998). These alterations can result from damage caused by *T. rangeli* to tissues and organs of the insects, such as modification of the Malpighian tubules, fat body, muscles, nerves, and tracheal cells (Watkins 1971a, b). Reproductive performance is also reduced in *R. prolixus* infected with *T. rangeli*. Infected adult females have delayed starts of oviposition after the first mating, and the conversion of the blood meal into eggs and hatching rates of laid eggs, are both decreased (Fellet et al. 2014). Environmental temperature was recently shown to be a modulator of the pathogenic effects of the *T. rangeli* Choachi strain in *R. prolixus*, as it apparently influences the development of the parasite in the hemolymph and in the salivary glands (Rodrigues et al. 2016). In vitro and in vivo experiments evaluating *T. rangeli* development in culture medium and in *R. prolixus* suggest that low (e.g., 21 °C) and high (e.g., 30 °C) temperatures affect negatively the parasite growth (Rodrigues et al. 2016). Temperature also differentially modulates the mortality rates of KP1(+) and KP1(−) *T. rangeli*-infected *R. prolixus*, with individuals infected with the KP1(−) strain having reduced mortality when kept at 40 °C (Hinestroza et al. 2016).

Effects of *T. rangeli* on the Behavior of Triatomines

Alterations to blood-feeding behavior are the most common behavioral change reported in hematophagous arthropods that transmit pathogens via saliva (Murdock et al. 2017). Such effects are also observed in *Rhodnius* species infected with *T. rangeli*. Difficulty in ingesting blood and increasing numbers of bites were reported in triatomines with salivary glands infected by *T. rangeli* (Grewal 1956; Tobie 1961; D'Alessandro and Mandel 1969; Añez and East 1984; Garcia et al. 1994; Cuba Cuba 1998). These alterations are probably the result of changes in the composition of proteins in the saliva. Infections with *T. rangeli*, particularly in individuals with high parasite densities, decrease the amount of salivary proteins, including hemeproteins (responsible for the cherry-red color of the glands) by ~60%, turning the glands to a whitish color (Paim et al. 2013). As saliva contains antihemostatic molecules that enable detection of host blood vessels, as well as a continuous flow of host blood (Ribeiro et al. 2004), a reduction of these molecules makes blood-feeding less efficient for *T. rangeli*-infected triatomines (Grewal 1956; Tobie 1961; D'Alessandro and Mandel 1969; Añez and East 1984; Garcia et al. 1994). In addition to alterations in blood-feeding behavior, a recent work has evaluated negative phototaxis, as well as spontaneous locomotory activity of *R. prolixus* infected intracoelomically with *T. rangeli* (Marliére et al. 2015). In this study,

infected nymphs have decreased negative phototaxis, becoming exposed to the light for longer periods than uninfected conspecifics. The infected nymphs also showed an increase in general locomotory activity, particularly during the photophase. Both results suggest that *T. rangeli*-infected insects would be more exposed to predation. Interestingly, infection by this parasite decreased the expression of the *Rpfor* gene in unfed nymphs (Marliére et al. 2015). This gene has been related to the modulation of locomotion in different groups of insects (de Belle et al. 1989; Ben-Shahar 2005; Ingram et al. 2005). Whether the parasite gains some advantage by changing the behavior of its vector is an interesting question to be addressed in future studies.

Effects of *T. rangeli* on Triatomine Immunity

Trypanosoma rangeli triggers a series of immunological responses during its development in the intestinal tract of its triatomine host. Besides causing changes in the species composition of the triatomine microbiota (see below), the presence of *T. rangeli* modifies the redox state of triatomines through increasing superoxide dismutase activity and decreasing the activity of enzymes related to hydrogen peroxide and hydroperoxide metabolism, which appear to affect parasite development (Cosentino-Gomes et al. 2014). *T. rangeli* infection also affects tissue-specific and time-dependent expression of nitric oxide synthase and nitrite production, which are altered differently in different tissues by parasite development (Whitten et al. 2007). On the other hand, the production of antimicrobial proteins—including lysozymes A and B, and prolixicin—is decreased in the intestine of *T. rangeli*-infected triatomines (Vieira et al. 2015). Interestingly, the presence of *T. rangeli* in the intestinal tract decreases immune responses in the hemolymph, suggesting that the trypanosome, while in the intestine, induces a generalized immunosuppression in triatomines. Oral infection of *R. prolixus* with the *T. rangeli* H14 strain inhibited the activation of the pro-phenoloxidase system (Gomes et al. 2003), and reduced the development of hemocyte microaggregates in the hemolymph (Garcia et al. 2004) when the triatomines were intracelomically inoculated with trypanosomes 5 days after the initial oral infection. In another study in which *R. prolixus* were orally infected, hemocyte phagocytosis rates were decreased (Figueiredo et al. 2008). When *T. rangeli* is directly injected into the coelomic cavity of *R. prolixus*, there is an initial increase in the number of hemocytes at the time when short epimastigotes are predominant. After a few days, the hemocytes are mostly clumped, and short epimastigotes are replaced by long epimastigotes (Takle 1988; Mello et al. 1995). The pro-phenoloxidase system is activated just after *T. rangeli* is injected into the hemolymph, but activity declines after initial activation, in parallel with the increase in the trypanosome population (Mello et al. 1995). Apparently, short but not long epimastigotes induce the activation of the pro-phenoloxidase system (Gomes et al. 1999). Short epimastigotes are also associated with the mobilization of proteases from the fat body into the hemolymph, which may be related to the activation of the pro-phenoloxidase system associated with these trypanosome forms (Feder et al. 1999). Lectins present in the hemolymph of *R. prolixus* have also been shown to

reduce the motility of *T. rangeli* epimastigotes in in vitro experiments, suggesting that these triatomine proteins can participate in insect cellular defenses (Mello et al. 1999).

Triatomine immune responses possibly vary according to different *T. rangeli* strains, which would partially explain variation observed in parasite development and pathogenicity in the studies published to date. For example, in a study that compared the generation of $\cdot O_2^-$ radicals and the activation of the pro-phenoloxidase system by the *T. rangeli* strains H14 and Choachi, greater immune responses were observed in triatomines infected with the H14 strain, which eliminated short epimastigotes from the hemolymph, and restricted the development of long epimastigotes, which were unable to invade the salivary glands. Trypanosomes from Choachi strain, which triggered milder immune responses, completed their development producing metacyclic forms (Whitten et al. 2001).

Interaction of *T. rangeli* and the Microbiota of Triatomines

As mentioned above, *T. rangeli* develops in all regions of the intestinal tract of triatomines, depending on the availability of nutritional resources. Consequently, the parasite has a close association with the triatomine microbiota, especially in the anterior midgut, where microorganisms are especially abundant (Wigglesworth 1936). The effects of *T. rangeli* on the mutualistic symbiont of *R. prolixus* seem to be an important factor determining the pathogenicity of this parasite to triatomines. The growth of bacteria on agar plates was inhibited by the presence of *T. rangeli* in trypanosomes obtained from in vitro culture and in those isolated from the contents of the midgut of experimentally infected *R. prolixus* (Watkins 1971a). In *R. prolixus* and *T. infestans*, the development of their respective mutualistic symbionts was differentially affected by co-infection with the Colombian Dr. Gonzáles *T. rangeli* strain: while the numbers of *R. rhodnii* were significantly reduced in *T. rangeli*-infected *R. prolixus*, no changes were evident in the mutualistic symbiont *R. triatomae* during *T. rangeli* infection of *T. infestans* (Eichler and Schaub 2002). The number of culturable bacteria was also significantly reduced in *R. prolixus* infected with the *T. rangeli* Macias strain (Vieira et al. 2015). Sequencing of the *16S rRNA* gene from samples of *T. rangeli*-infected triatomines showed that trypanosomes altered the bacterial communities present in the anterior midgut, which included a decrease in the amount of Enterococcaceae and Mycobacteriaceae, and an increase in Burkholderiaceae (Vieira et al. 2015). In addition, *T. rangeli* showed antibacterial activity against *S. marcescens*, *Staphylococcus aureus* and *Escherichia coli* (Vieira et al. 2015). It is possible that reduction in the population size of the mutualistic symbionts affects the intestine environment and facilitates access of *T. rangeli* to the hemocoel.

5 Conclusions and Open Questions

The microbiota of triatomine varies between triatomines from laboratory colonies and those obtained from the field, and also between specimens sampled from the same location. Shotgun metagenomic sequencing in addition to culture-dependent methodologies can improve identification of mutualistic symbionts, since there are currently only four species known for triatomines. The factors enabling the apparently paradoxical high densities of mutualistic symbionts in the anterior midgut, which has high levels of antibacterial activity, but low densities of these symbionts in the posterior midgut, which has low antibacterial activity, are important questions requiring further research.

In order to completely understand the development of *T. cruzi* in the different regions of the triatomine intestine, some questions still require further investigation. Regarding the development of *T. cruzi* in the anterior midgut, the use of new techniques, such as fluorescence microscopy, bioluminescence imaging, and qPCR, as well as comparisons between different trypanosome strains and triatomine species, will provide more information about the colonization process. With regard to the development of epimastigotes in the rectum, the molecular components within the hydrophobic attachment zone of the flagellum (Schmidt et al. 1998) remain to be identified. The conditions in the rectum (e.g., concentrations of oxygen, free amino acids, proteins, and lipids) and their effects on *T. cruzi* will allow the correlation and comparison of the results obtained for in vitro metacyclogenesis with findings from in vivo studies.

Investigations of *T. rangeli* are complicated by the variable proportion of intestinal infections that become systemic. Although injecting parasites into the hemocoel is not the natural route of infection, it facilitates the study of the development of this parasite in the hemolymph and salivary glands. The *R. prolixus*-*T. rangeli* combination appears to be a good model for studies of invertebrate immunology, and should enable exploration of vector cellular responses to parasite infection.

In general, more detailed investigations on the development of trypanosome strains belonging to different lineages, but originating from the same locations, would be desirable. The use of metagenomic, proteomic, and metabolomic data will significantly improve the comprehension of the processes that drive these parasite-microbiome-vector systems.

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The Ecology and Natural History of Wild Triatominae in the Americas

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Abstract Triatomine bugs, the vectors of Chagas disease, feed primarily on vertebrate blood and live in close association with their hosts. Here we provide an updated synthesis of current knowledge about the ecology and natural history of the 140+ American triatomine-bug species. We suggest that the bugs' highly diverse lifestyles fall into two major classes. "Sit-and-wait" nest specialists are associated with the nests of particular hosts – on which the bugs feed almost ectoparasitically. Active-foraging, "stalker" host generalists, in contrast, occupy certain discrete microhabitats (rock outcrops, trees, palm crowns, etc.) and feed opportunistically on the potentially diverse vertebrates that also use those microhabitats. Within each foraging-lifestyle class, triatomines have adapted to widely diverse ecoregions, from deserts to rainforests, and habitats, from underground to forest canopy. About half of all living species are arboreal and about half are terrestrial. All likely descend, however, from a tree-dwelling, host-generalist "stalker" ancestor; the "sit-and-wait," nest-specialized lifestyle independently evolved several times to yield ~30% of extant taxa. Foraging-related adaptations may have contributed significantly to shaping the morphological, physiological, and behavioral diversity of the bugs. From a practical standpoint, we note that the most dangerous domestic vectors of *Trypanosoma cruzi* (*Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiata*) are opportunistic "stalkers" – i.e., bugs that were preadapted to feed on diverse hosts in shared microhabitats. We expect that, by introducing a fresh perspective on triatomine-bug ecology and behavior, our "foraging-lifestyle hypothesis" will open new research avenues and will thus, ultimately, contribute to the development of improved strategies for the prevention of vector-borne Chagas disease.

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1 Introduction

1.1 The Triatominae

The Triatominae are true bugs that feed primarily on vertebrate blood. In particular, triatomines usually require at least one blood meal to molt from each post-hatching developmental stage to the next (Lent and Wygodzinsky 1979). The shift from the ancestral invertebrate-preying habit to a derived blood-feeding habit triggered the evolution of a host of ecological, behavioral, physiological, and morphological adaptations (e.g., Lent and Wygodzinsky 1979; Lazzari and Lorenzo 2009; Hwang and Weirauch 2012; Lazzari et al. 2013; Mesquita et al. 2015; Barrozo et al. 2017; Monteiro et al. 2018). Here, we emphasize two key consequences of blood feeding. First, triatomines act as the vectors of *Trypanosoma cruzi*, a blood/tissue parasite of mammals that causes severe human disease – Chagas disease (Rassi Jr et al. 2010). Second, triatomine bugs live in close association with the hosts they feed upon in shared, discrete "microhabitats" – burrows, rock outcroppings, trees, palms, nests, bromeliads, and so on (Lent and Wygodzinsky 1979; Miles 1979). This review is about such microhabitats and hosts.

Although eight *Triatoma* and six *Linshcosteus* species are native to the Old World, vector-mediated transmission of *T. cruzi* is restricted to the Americas – the region where triatomines first evolved and where they diversified most profusely (Monteiro et al. 2018). This chapter will therefore focus on New World-native triatomines – i.e., over 90% of all known species. We will summarize and update current knowledge on the natural habitats of American triatomines, emphasizing associations with vertebrate hosts and considering also microhabitat segregation/stratification (from underground burrows to tree-canopy epiphytes) across broad biogeographic units. We will follow the systematic-biogeographic arrangement of Monteiro et al. (2018). To the extent possible given data availability, we will also comment on the discrete typing units (DTUs; Zingales et al. 2009) of *T. cruzi* that have been found circulating among bugs and mammals in specific microhabitats.

1.2 Foraging Lifestyles in the Triatominae: "Sit-and-Wait" Nest Specialists vs. "Stalker" Host Generalists

In this review we suggest that the highly diverse ways in which triatomines relate to their microhabitats and hosts can be usefully operationalized by defining two major "foraging-lifestyle classes" (Box 1). Nest-specialist triatomine-bug species largely behave as "sit-and-wait" foragers associated with the nests (including burrows, dens, lodges, "houses," "beds," and other more or less permanent and complex

breeding or resting structures) of specific hosts – on which the bugs, therefore, feed preferentially. "Sit-and-wait" triatomines live and forage within their hosts' nests much in the way cimicids including bedbugs live and forage within the "nests" (including human beds or bat roosts) of their vertebrate hosts (Box 1). Active-foraging, opportunistic host-generalist bug species, on the other hand, stably occupy, and breed in, discrete microhabitats (e.g., a rock outcropping, a tree, or a palm crown) irrespective of whether their vertebrate hosts actually nest there; the bugs, therefore, feed on the potentially diverse hosts that make use (for, e.g., resting, foraging, or, at times, breeding) of the shared microhabitat. These host-generalist triatomines largely behave as "stalker" micropredators that "hunt" their hosts much in the way many predatory reduviids hunt invertebrate prey in open, non-nest microhabitats (Box 1).

Box 1 Traits associated with foraging-lifestyle classes in the Triatominae: a proposal

Traits	Foraging strategy/lifestyle	
	"Sit-and-wait"	"Stalk"
Primary traits[a]		
Breeding microhabitat	Almost invariably a nest	Usually non-nest
Host seeking	Largely passive[b]	Active[c]
Feeding	Virtually ectoparasitic	Opportunistic
Host selectivity	Specialist	Generalist
Other traits[d]		
Body size	Smaller	Larger
Head shape	Stouter	More streamlined
Movements	Slower	Swifter
Sensory system[e]	Simplified	More complex
Saliva chemistry	More highly specialized	Less specialized
Blood meal size	Smaller	Larger
Eggs laid per female	Less	More
Bites to humans	Painful, strongly allergenic	Painless, weakly allergenic
Dwelling infestations[f]	Less common, lighter	More common, heavier
Dwelling foci[f]	Clustered on beds, nests, etc.	Scattered across refuges

[a]Used to define our two broad classes
[b]Much in the way cimicids (including *Cimex* bedbugs) live and forage within the nests (including human beds or bat roosts) of their vertebrate hosts
[c]Much in the way most predatory reduviids (the "assassin bugs") live and forage in open, non-nest microhabitats
[d]We regard these as plausible hypotheses that can in principle be tested – using, importantly, suitable approaches for controlling for phylogenetic relatedness and other sources of non-independence
[e]More specifically, the parts of the sensory system involved in the complex, risky process of feeding on the blood of vertebrate hosts
[f]By species able to breed in man-made habitats

Although some triatomine-bug species may use both foraging strategies, we propose that most can be viewed as primarily "sit-and-wait" nest specialists or primarily "stalker," opportunistic host generalists – where "primarily" is meant to reflect the relative frequency of each type of strategy and also suggests a role for adaptive evolution in shaping species- or, more broadly, lineage-specific lifestyle and behavior patterns.

In articulating this idea, it is important to note that wingless triatomine-bug nymphs tend to remain in the microhabitat they are born in. For some species, this is almost invariably a vertebrate nest (in the broad sense given above), and these species are more likely to live as "sit-and-wait" nest specialists (Box 1). Most *Panstrongylus*, for example, breed within "closed" microhabitats (tree holes, underground burrows) used by nesting vertebrates. There, the nymphs live in such close physical proximity to their nesting hosts that they barely need to move to get a blood meal – they can behave as (virtually ectoparasitic) "sit-and-wait" foragers (Box 1). *Paratriatoma hirsuta*, several woodrat nest-associated North American *Triatoma* species, the *Psammolestes*, and *Rhodnius paraensis* match this profile too. This type of behavior also results in the bugs, and particularly nymphs, overall appearing as rather host-specialized – they feed most often on the hosts whose nests they infest (Box 1). Some nest-dwelling triatomines, however, may feed both on the nesting host and on the nest-associated fauna including invertebrates; *Microtriatoma trinidadensis* seems to be an example. The two known species of *Cavernicola* are tightly associated with a particular kind of "nest" – bat roosts.

Most triatomine-bug species, on the other hand, breed in more "open" (non-nest) microhabitats (rock outcroppings, palm crowns, bromeliads, or tree-trunk surfaces) and tend to feed opportunistically on available hosts (Box 1). These bugs, including nymphs, usually need to actively search through their microhabitat to locate hosts and get blood meals. This defines the typical behavior of active-foraging, "stalker," opportunistic host generalists – a behavior that also results in the bugs appearing as overall less host-specialized in their feeding preferences (Box 1). *Dipetalogaster*, *Mepraia*, *Hermanlentia*, most *Triatoma*, most *Rhodnius*, and probably most Bolboderini, overall match this profile. If, however, a vertebrate builds a nest in a microhabitat occupied by a "stalker" species colony, the odds are high that the bugs will exploit it; palm *Rhodnius* colonies, for example, often grow denser when they share their palm-crown habitat with nesting vertebrates.

In contrast to wingless nymphs, adult triatomines have (with few exceptions) functional wings. When adult bugs leave their breeding site to disperse across the landscape (motivated by hunger or other stimuli), they may behave as typical "stalkers" even if they grew up as typical "sit-and-wait" nest specialists. Thus, *Panstrongylus lignarius* nymphs grow inside hollow-tree mammal nests, yet adults may forage on the external surface of trees. As a more extreme example, an adult, *T. cruzi*-infected *Panstrongylus geniculatus* was recorded landing on a light-trap cloth and feeding upon an *Eacles* moth in the Guiana lowland moist forests (Garrouste 2009). If, on the other hand, a dispersing host-generalist "stalker" finds a suitable nest or burrow, the odds are high that it will exploit it; several putative "stalker" *Triatoma* species, for example, use armadillo burrows on occasion, and

Rhodnius neglectus often infests vertebrate nests on the crowns of *Mauritia flexuosa* palms – which otherwise are probably low-quality habitat for the species. Importantly, this "disperse-and-feed" adult-bug behavior is characteristic of the many species that are recorded invading human dwellings – hungry bugs, whether "sit-and-wait" nest specialists or "stalker," opportunistic host generalists, will attempt to feed on the hosts that are available at the sites (houses, chicken coops, corrals, etc.) they land on. For reasons that are not well understood, only some of those bugs, however, will succeed at establishing viable breeding colonies (Abad-Franch et al. 2010).

Below we will highlight in ***bold italics*** the name of each triatomine-bug species the first time it appears in the *Section* (i.e., foraging-lifestyle/microhabitat combination) where we believe it primarily belongs. Next to the name we indicate, in square brackets, whether the species has been (i) found naturally infected with *T. cruzi* [Tc] (or not [−]); (ii) caught at light (in light traps or houses) [L]; and (iii) reported from human dwellings (houses or annex structures) [H]. For example, ***Paratriatoma hirsuta*** [−/L/H] indicates that we found no records of *T. cruzi* infection for *Pa. hirsuta*, which has been caught at light and in human dwellings. We finally note that the natural habitats and hosts of several triatomine-bug species remain unknown; in those cases, we tentatively classify each species in a group based on its systematic/phylogenetic affinities (largely following Monteiro et al. 2018) and, when available, on data on the ecology and behavior of non-wild populations – including, e.g., microhabitat-use or host-selection patterns. We regard these tentative classifications as specific hypotheses that may stimulate and guide future field research.

2 "Sit-and-Wait" Nest Specialists

2.1 Underground Nests

2.1.1 Armadillo Burrows

Panstrongylus geniculatus [Tc/L/H] is widespread across moist/forested and dry/open ecoregions from southern Mexico to northern Argentina (Monteiro et al. 2018). In dry-to-arid ecoregions, *P. geniculatus* closely associates with armadillo burrows, whereas in moist forests it also exploits mammal (most often rodent) nests inside fallen logs, tree-root cavities, or (usually ground level) tree holes (see Sect. 2.3.2). *Panstrongylus geniculatus* has also been found with underground-burrowing *Cuniculus* pacas, in caves with roosting bats, and, on occasion, in bromeliads and palms (Tables 1 and 2). Apart from armadillos, rodents, and bats, this species may feed on marsupials (*Didelphis*, *Philander*), woolly monkeys, tamarins, sloths, anteaters, tayras, birds, and arthropods. About 96% (score 95% confidence interval [CI_{95}] 94–97%) of ~700 *T. cruzi* stocks from *P. geniculatus* were characterized as DTU TcI, with only 3% TcIII and a few TcIV. Most of those bugs, however, were caught in northern Venezuela, where TcI circulates extensively; excluding those

samples, 31% (CI_{95} 20–46%) of 45 *T. cruzi* from *P. geniculatus* were TcIII, 60% (CI_{95} 46–73%) were TcI, and 9% were TcIV (Lent and Wygodzinsky 1979; Miles 1979; Garrouste 2009; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Patterson et al. 2009; Omah-Maharaj 1992; Molinari et al. 2007; Abad-Franch et al. 2015; Hernández et al. 2016; Carrasco et al. 2012, 2014; Batista 2018; Batista et al. 2019).

Panstrongylus lutzi [Tc/L/H] is a second species that might be primarily associated with armadillo burrows; it lives in the semiarid Caatinga shrubland of northeastern Brazil, where adult bugs have also been found in small hollow trunks of *Auxemma* trees and among rocks with nesting rodents. The few *P. lutzi*-derived *T. cruzi* stocks characterized so far were all TcIII (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Patterson et al. 2009; Dias-Lima et al. 2003; Garcia et al. 2005; Câmara et al. 2010).

In contrast to the results from *P. geniculatus* mentioned above, over 94% (CI_{95} 86–97%) of *T. cruzi* stocks from armadillos (*Dasypus*, *Euphractus*, *Chaetophractus*, and unidentified specimens) have been characterized as TcIII, although infections with TcI, TcII, TcIV, and TcV have also been reported (Brenière et al. 2016; Izeta-Alberdi et al. 2016).

We finally note that *Panstrongylus rufotuberculatus*, *Panstrongylus megistus*, and *Panstrongylus guentheri* (Sect. 2.3.2), as well as 22 putative host-generalist "stalker" species in *Eratyrus* and *Triatoma*, have all been reported as occasional armadillo-burrow users (Table 1). Although this association seems unlikely for palm-dwelling *Rhodnius* (Sect. 3.2.2), it has been suggested that several species in the genus may sporadically feed on armadillos (cf. Georgieva et al. 2017; Rabinovich et al. 2011) (Table 1).

2.1.2 Other Underground Burrows

Populations of *Triatoma recurva*, *Triatoma gerstaeckeri*, *Triatoma eratyrusiformis*, and *Triatoma patagonica* may associate with underground rodent or rabbit burrows in semiarid-to-arid ecoregions; these species, however, are putative host-generalist "stalkers" that also feed on other hosts outside the burrows' tunnels and chambers (Sect. 3.1.2). The primarily arboreal, opportunistic host-generalist "stalker" *Rhodnius prolixus* has also been recorded with underground-burrowing pacas (*Cuniculus paca*) (Sect. 3.2.2); finally, *Panstrongylus geniculatus* and *Panstrongylus megistus* may occur in the burrows of, respectively, *Cuniculus* pacas and *Dasyprocta* agoutis (Sects. 2.1.1 and 2.3.2).

Table 1 The foraging lifestyles and natural habitats/microhabitats of wild Triatominae in the Americas

Lifestyle	Habitat	Microhabitat	Primary association	Microhabitat users[a]
"Sit-and-wait" nest specialists	Underground burrows	Armadillo burrows	*P. geniculatus*, *P. lutzi*	*T. sanguisuga*, *T. gerstaeckeri*, *T. dimidiata* I, *T. mazzottii*, *T. pallidipennis*, *T. longipennis*, *T. nigromaculata*, *T. maculata*, *T. vitticeps*, *T. eratyrusiformis*, *T. brasiliensis*, *T. juazeirensis*, *T. lenti*, *T. melanica*, *T. sordida*, *T. vandae*, *T. costalimai*, *T. guasayana*, *T. rubrovaria*, *T. patagonica*, *E. cuspidatus*, *P. megistus*, *P. rufotuberculatus*, *P. guentheri*; (*T. dimidiata* II); [*R. pallescens*, *R. brethesi*, *R. prolixus*, *R. neglectus*]
		Other underground burrows	(None known)	*T. incrassata*, *T. lecticularia*, *T. recurva*, *T. gerstaeckeri*, *T. eratyrusiformis*, *T. patagonica*, *P. geniculatus*, *P. megistus*, *R. prolixus*
	Ground nests	Woodrat nests	*T. protracta*, *T. lecticularia*, *Pa. hirsuta*, *T. incrassata*, *T. peninsularis*, *T. sinaloensis*, *T. rubida*, *T. neotomae*, *T. sanguisuga*, *T. indictiva*; (*T. barberi*, *T. nitida*)	*D. maxima*, *T. recurva*, *T. gerstaeckeri*, *T. pallidipennis*, *T. mazzottii*, *T. phyllosoma*
		Other mammal ground nests	*T. rubrofasciata*[b]; (*Bo. scabrosa*, *P. tupynambai*)	*T. sanguisuga*, *T. longipennis*, *T. pallidipennis*, *T. phyllosoma*, *T. picturata*, *M. spinolai*, *T. eratyrusiformis*, *T. breyeri*, Andean *T. infestans*, *T. sordida*, *T. patagonica*, *T. platensis*, *T. flavida*/*bruneri*, *P. geniculatus*, *P. howardi*, *P. megistus*, *R. pallescens*; (*T. barberi*, *T. confusa*, *T. oliveirai*)
	Arboreal nests	Arboreal bird nests	*Ps. arthuri*, *Ps. tertius*, *Ps. coreodes*, *T. platensis*, *T. delpontei*	*T. sanguisuga*, *T. dimidiata* I, Chacoan *T. infestans*, *T. sordida*, *T. guasayana*, *T. maculata*, *T. pseudomaculata*, *P. lignarius*, *P. geniculatus*, *P. howardi*, *P. megistus*, *P. guentheri*, *R. pallescens*, *R. ecuadoriensis* I, *R. pictipes*, *R. prolixus*, *R. neglectus*, *R. nasutus*, *A. malheiroi*; (*T. nigromaculata*, *R. ecuadoriensis* II)

(continued)

Table 1 (continued)

Lifestyle	Habitat	Microhabitat	Primary association	Microhabitat users[a]
		Arboreal mammal nests	*R. paraensis*, *R. domesticus*/*zeledoni*, southern *R. ecuadoriensis* I, *P. megistus*, *P. lignarius*, *P. rufotuberculatus*, *Mi. trinidadensis*, *Mi. borbai*, *Pb. carioca*; (*P. humeralis*, *P. mitarakaensis*, *P. martinezorum*, *P. howardi*, *P. chinai*, *P. guentheri*, *P. diasi*, *P. lenti*, *R. ecuadoriensis* II)	*T. lecticularia*, *T. sanguisuga*, *T. dimidiata* I, *T. maculata*, *T. pseudomaculata*, *T. sordida*, *T. guasayana*, Chacoan *T. infestans*, *T. tibiamaculata*, *P. geniculatus*, *P. lutzi*, *R. pallescens*, *R. pictipes*, *R. stali*, *R. prolixus*, *R. neglectus*/*milesi*, *R. nasutus*, *B. herreri*, *B. peruvianus*, *Pb. yurupucu*; (*T. barberi*, *T. dimidiata* II)
	Bat roosts	Bat roosts	*C. pilosa*, *C. lenti*	*T. rubida*, rock *T. brasiliensis*, *E. mucronatus*, *E. cuspidatus*, *P. geniculatus*, *P. rufotuberculatus*; (see also "caves" and "trees" below)
Host-generalist "stalkers"	Terrestrial	Caves	*T. mopan*, cave *T. dimidiata* I, *H. matsunoi*	*T. nitida*, *T. carrioni*, *E. mucronatus*, *T. gerstaeckeri*, *T. hegneri*, *T. mazzottii*, *T. longipennis*, *T. pallidipennis*, *T. picturata*, rock *T. brasiliensis*, *T. baratai*, *T. patagonica*, *T. flavida*/*bruneri*, *P. geniculatus*; (*T. obscura*, *T. confusa*)

Lifestyle	Habitat	Microhabitat	Primary association	Microhabitat users[a]
		Rocks and stones	*D. maxima*, *T. gerstaeckeri*, *T. dimidiata* I, *T. hegneri*, *T. recurva*, *T. longipennis*, *T. picturata*, *T. pallidipennis*, *T. mazzottii*, *T. phyllosoma*, *M. gajardoi*, *M. parapatrica*, *M. spinolai*, *T. eratyrusiformis*, *T. breyeri*, *T. vitticeps*, Andean *T. infestans*, rock *T. brasiliensis*, *T. juazeirensis*, *T. sherlocki*, *T. lenti*, *T. bahiensis*, *T. petrocchiae*, *T. melanica*, *T. arthurneivai*, *T. wygodzinskyi*, *T. costalimai*, *T. jatai*, *T. rubrovaria*, *T. circummaculata*, *T. carcavalloi*, *T. pintodiasi*, *T. oliveirai*, *T. klugi*, *T. limai*, *T. patagonica*, *T. jurbergi*, *T. vandae*, *T. boliviana*; (*T. mexicana*, *T. dimidiata* II, *T. huehuetenanguensis*, *T. bassolsae*, *T. brailovskyi*, *T. gomeznunezi*, *T. guazu*, *T. baratai*, *T. williami*, *T. deaneorum*, *T. matogrossensis*, *T. flavida/bruneri*, *T. confusa*, *T. obscura*)	*T. lecticularia*, *T. sanguisuga*, *T. guasayana*, *T. sordida*, *E. cuspidatus*, *P. chinai*, *P. megistus*, *P. lutzi*, *P. tupynambai*; (*T. carrioni*, *T. indictiva*, *T. melanocephala*, *E. mucronatus*, *P. howardi*)
		Terrestrial plants	Cactus *T. brasiliensis*; (*T. melanocephala*)	*T. gerstaeckeri*, *T. sanguisuga*, *T. dimidiata* I, *M. spinolai*, *T. eratyrusiformis*, *T. maculata*, *T. pseudomaculata*, *T. infestans*, *T. sordida*, *T. guasayana*, *T. patagonica*, *P. geniculatus*, *P. howardi*, *P. megistus*, *R. ecuadoriensis* I, *R. neglectus/milesi*, *R. domesticus/zeledoni*, *Bo. scabrosa*; (*T. phyllosoma*)
	Arboreal	Trees	*T. dispar*, *T. nigromaculata*, *T. carrioni*, *T. ryckmani*, *T. maculata*, *T. pseudomaculata*, *T. sordida*, *T. garciabesi*, Chacoan *T. infestans*, *T. guasayana*, *E. mucronatus*, *B. costaricensis*, *B. herreri*, *B. laportei*, *B. peruvianus*; (*T. venosa*, *T. bolivari*, *E. cuspidatus*, *B. rugulosus*, *B. corredori*, *B. ferroae*, *B. pittieri*, *A. goyovargasi*, *A. malheiroi*)	*T. lecticularia*, *T. sanguisuga*, *T. indictiva*, *T. longipennis*, *T. picturata*, *T. eratyrusiformis*, *T. tibiamaculata*, *P. geniculatus*, *P. lignarius*, *P. humeralis*, *P. rufotuberculatus*, *P. megistus*, *P. lutzi*, *R. pallescens*, *R. ecuadoriensis* II, *R. pictipes*, *R. neivai*, *R. prolixus*, *R. neglectus/milesi*, *R. nasutus*, *Mi. trinidadensis*, *Pb. carioca*; (*T. dimidiata* I/II, *T. huehuetenanguensis*, *P. mitarakaensis*, *R. domesticus/zeledoni*)

(continued)

Table 1 (continued)

Lifestyle	Habitat	Microhabitat	Primary association	Microhabitat users[a]
		Palms	*R. pallescens*, *R. colombiensis*, northern *R. ecuadoriensis* I, *R. pictipes*, *R. stali*, *R. brethesi*, *R. neivai*, *R. prolixus*, *R. robustus* I, *R. robustus* IV, *R. robustus* V, *R. marabaensis*, *R. montenegrensis*, *R. barretti*, *R. dalessandroi*, *R. neglectus/milesi*, *R. nasutus*, *Rhodnius* sp. *aff. nasutus*; (*R. amazonicus*, *Rhodnius* sp. *aff. pictipes*)	*T. dimidiata* I, *T. sanguisuga*, *T. maculata*, *T. pseudomaculata*, *T. infestans*, *T. platensis*, *T. sordida*, *E. cuspidatus*, *E. mucronatus*, *T. tibiamaculata*, *P. geniculatus*, *P. lignarius*, *P. rufotuberculatus*, *P. howardi*, *P. megistus*, *R. domesticus/zeledoni, A. malheiroi*, *B. herreri*, *B. rugulosus*, *C. pilosa*, *Mi. trinidadensis*, *Mi. borbai*, *Pb. carioca*; (*T. dimidiata* II, *T. huehuetenanguensis*)
		Epiphytes	*T. tibiamaculata*; (*Pb. yurupucu*)	*T. ryckmani*, *T. carrioni*, *T. maculata*, *T. sordida*, *T. garciabesi*, Chacoan *T. infestans*, *T. guasayana*, *P. geniculatus*, *P. megistus*, *R. pictipes*, *R. prolixus*, *R. robustus* IV, *R. domesticus/zeledoni*, *M. trinidadensis*, *M. borbai*, *B. costaricensis*

Generic abbreviations: *A. Alberprosenia*, *B. Belminus*, *Bo. Bolbodera*, *C. Cavernicola*, *D. Dipetalogaster*, *E. Eratyrus*, *H. Hermanlentia*, *M. Mepraia*, *Mi. Microtriatoma*, *P. Panstrongylus*, *Pa. Paratriatoma*, *Pb. Parabelminus*, *Ps. Psammolestes*, *R. Rhodnius*, *T. Triatoma*

Species in round brackets are likely to be associated with the microhabitat; the reported association with armadillos seems unlikely for *Rhodnius* species (in square brackets)

We treat *R. milesi* as a synonym of *R. neglectus* and consider that *R. zeledoni* is in all likelihood a synonym of *R. domesticus* (Monteiro et al. 2018)

Note that populations of *T. dimidiata* I, *T. infestans*, *T. brasiliensis*, and *R. ecuadoriensis* I seem to have adapted to wild microhabitats other than their primary ones; these are the only species appearing more than once in the "primary association" column. See text for further details

[a]Species that can make more or less sporadic use of each habitat and microhabitat; not all species in each line will match the "lifestyle" column

[b]Non-native populations associated with ship rats (*Rattus rattus*) (Monteiro et al. 2018)

Table 2 Palms and triatomine bugs in the Americas

Palm	Primarily palm-dwelling species	Occasional users
Native		
Attalea cohune	–	*T. dimidiata* I/II; (*T. huehuetenanguensis*)
A. butyracea	*R. prolixus*, *R. robustus* I, *R. montenegrensis*, *R. barretti*, *R. pallescens*, *R. colombiensis*, *R. pictipes*, *R. neivai*; (*R. robustus* IV)	*T. maculata*, *T. dimidiata* I, *P. lignarius*, *E. mucronatus*, *E. cuspidatus*
A. speciosa	*R. robustus* V, *R. montenegrensis*, *R. neglectus/milesi*, *R. nasutus*, *R. pictipes*; (*R. marabaensis*)	*T. sordida*, *T. pseudomaculata*, *P. lignarius*, *P. megistus*, *Mi. trinidadensis*
A. maripa	*R. prolixus*, *R. robustus* IV, *R. montenegrensis*, *R. neglectus/milesi*, *R. pictipes*; (*R. robustus* V, *R. marabaensis*)	*P. lignarius*, *Mi. trinidadensis*[a]
A. dubia	–	*Pb. carioca*[a]
A. phalerata	*R. montenegrensis*, *R. neglectus*, *R. stali*; (*R. marabaensis*)	*T. sordida*, *P. megistus*
A. oleifera	*R. neglectus/milesi*	–
Attalea sp.	*R. neivai*	*R. domesticus/zeledoni*, *T. tibiamaculata*[b], *P. megistus*, *E. cuspidatus*
Acrocomia aculeata	*R. neglectus/milesi*, *R. pictipes*, *R. nasutus*, *R. prolixus*, *R. robustus* I, *R. pallescens*; (*R. robustus* IV, *R. marabaensis*)	*Ps. tertius*[c], *T. sordida*, *T. maculata*, *T. infestans*, *P. lignarius*, *P. megistus*, *P. geniculatus*, *E. cuspidatus*, *Mi. trinidadensis*[a]
Aiphanes eggersii	–	*P. howardi*
Astrocaryum aculeatum	(*R. robustus* IV, *R. montenegrensis*)	–
As. murumuru	*R. pictipes*, *R. stali*; (*R. robustus* IV, *R. montenegrensis*)	–
Astrocaryum spp.[d]	*R. pictipes*; (*R. montenegrensis*, *R. barretti*)	–
Butia yatay	–	*T. sordida*, *T. platensis*, *Ps. coreodes*
Bactris sp.	–	*B. herreri*
Cocos nucifera	*R. prolixus*, *R. pallescens*	*T. maculata*
Copernicia alba	*R. stali* (as *R. pictipes*)	*T. sordida*
Co. prunifera	*R. nasutus*, *R. neglectus/milesi*	*Ps. tertius*, *T. sordida*, *T. pseudomaculata*
Co. tectorum	*R. prolixus*, *R. neivai*, *R. pallescens*	*T. maculata*, *P. geniculatus*
Copernicia sp.	–	*P. geniculatus*
Elaeis oleifera	*R. pallescens*	*P. geniculatus*, *E. cuspidatus*
Euterpe oleracea	–	*A. malheiroi*[e]
Leopoldinia piassaba	*R. brethesi*; [*R. prolixus*]	*P. geniculatus*

(continued)

Table 2 (continued)

Palm	Primarily palm-dwelling species	Occasional users
Mauritia flexuosa	*R. neglectus*/*milesi*[a,c], *R. nasutus*[c], *R. prolixus*[c]; (*R. robustus* IV)	*Ps. tertius*[c], *T. sordida*[c], *P. lignarius*[f], *M. borbai*[a], *C. pilosa*[g]
M. carana	(*R. robustus* IV)	*P. lignarius*
Oenocarpus bacaba	–	*A. malheiroi*[f]
Oe. bataua	*R. prolixus*, *R. dalessandroi*, *R. pictipes*, *R. stali*, *R. pallescens*, *R. barretti*; (*R. robustus* I, *R. robustus* IV, *R. montenegrensis*)	*T. maculata*
Oe. distichus	*R. neglectus*/*milesi*; (*R. marabaensis*)	–
Oenocarpus sp.	–	*B. rugulosus*
Phytelephas aequatorialis	*R. ecuadoriensis* I	*P. rufotuberculatus*
Ph. tenuicaulis	*R. pictipes*; (*R. montenegrensis*, *R. barretti*)	–
Sabal mauritiiformis	*R. prolixus*	–
Sa. palmetto	–	*T. sanguisuga*
Sabal sp.	–	*T. maculata*, *P. lignarius*
Syagrus coronata	–	*T. sordida*
S. oleracea	*R. neglectus*/*milesi*, *R. nasutus*	*T. sordida*, *P. megistus*
S. romanzoffiana	*R. neglectus*/*milesi*	*T. sordida*, *P. geniculatus*, *P. megistus*
S. schizophylla	–	*P. megistus*
Non-native		
Elaeis guineensis	*R. prolixus*, *R. pictipes*, *R. ecuadoriensis* I; (*R. montenegrensis*, *R. barretti*)	–
Livistona australis	*R. neglectus*/*milesi*	–
Roystonea borinquena	*R. neglectus*/*milesi*	–
R. oleracea	*R. neglectus*/*milesi*	–

Generic abbreviations (triatomine-bug Latin binomials): *A. Alberprosenia*, *B. Belminus*, *C. Cavernicola*, *E. Eratyrus*, *Mi. Microtriatoma*, *P. Panstrongylus*, *Pb. Parabelminus*, *Ps. Psammolestes*, *R. Rhodnius*, *T. Triatoma*

Species in round brackets are likely to be associated with the microhabitat; the reported association of *Rhodnius prolixus* with *Leopoldinia piassaba* is likely erroneous (in square brackets)

[a]*Didelphis* nest

[b]In palms tentatively identified as *Attalea burretiana* and *A. salvadorensis*

[c]Bird nests

[d]Tentatively identified as *Astrocaryum chambira* and *As. murumuru* var. *urostachys*

[e]Woodpecker nest inside the palm stem

[f]Hollow palm stems

[g]Bat roosts

We treat *R. milesi* as a synonym of *R. neglectus* and consider that *R. zeledoni* is in all likelihood a synonym of *R. domesticus* (Monteiro et al. 2018).

2.2 Ground Nests

2.2.1 Woodrat Nests

Most triatomine-bug species of the North American clade (sensu Monteiro et al. 2018, yet excluding *Triatoma indictiva*) are primarily associated with woodrat (mainly *Neotoma*) nests and behave as virtually ectoparasitic, "sit-and-wait" nest specialists (Table 1). Woodrats build their nests or lodges in stony microhabitats (rocky outcrops, cliffs, canyons); at the base of cacti, yucca clumps, or bushes; or in hollow logs. The nests usually include an aboveground heap of twigs, cactus fragments, stones, or even bones or litter, plus frequently (but not always) a system of underground tunnels and breeding chambers. These nests often remain occupied by resident woodrats year-round and over several generations, and may grow to large sizes (Nowak 1997). Within woodrat lodges, "many of the bugs will be found in the innermost grass nests" – i.e., in the burrows' breeding chambers (Usinger 1944, p. 9). Woodrat lodges can provide shelter to other vertebrates including skunks, quails, lizards, or rattlesnakes (Ryckman and Ryckman 1967; Ryckman 1954). For *Neotoma* taxonomy and distribution, we follow the Catalogue of Life (www.catalogueoflife.org) and the International Union for Conservation of Nature's Red List of Threatened Species (www.iucnredlist.org).

Triatoma protracta [Tc/L/H] associates with *Neotoma albigula*, *Neotoma fuscipes*, *Neotoma lepida*, and likely *Neotoma devia* and *Neotoma bryanti* – all from dry-to-arid ecoregions across the southwestern United States and northwestern Mexico. East of the Rocky Mountains-Sierra Madre range system, *T. protracta* occurs with *Neotoma micropus*. Most *T. cruzi* stocks from wild *T. protracta* have been characterized as TcI, but TcIV has also been reported. ***Triatoma lecticularia*** [Tc/H] infests *N. micropus* nests in the open landscapes of the central-southern United States and northeastern Mexico, where it may also associate with *Otospermophilus* rock squirrels; in forested regions to the east, *T. lecticularia* occurs also in hollow trees with *Sciurus* fox-squirrels and *Procyon* raccoons. Thirty-three of 52 *T. cruzi* stocks isolated from *T. lecticularia* were typed as TcIV; 19 were TcI. The only known hosts of ***Paratriatoma hirsuta*** [−/L/H] are dry ecoregion-adapted woodrats (*N. lepida* [plus likely *N. bryanti* and *N. devia*], *N. albigula*, and *N. fuscipes*); records with *N. mexicana* seem dubious. ***Triatoma incrassata*** [−] has been reported in association with *Neotoma* and *Otospermophilus* in the Chihuahua and Sonora deserts. ***Triatoma peninsularis*** [Tc/L] is only known from arid Baja California; records of this species in the nests of *N. lepida*, which does not occur in that region, likely refer to nests of *N. bryanti*. ***Triatoma sinaloensis*** [Tc] is probably closely related to *T. peninsularis* and has been found in nests of *Neotoma phenax* and *N. albigula* along the dry Sonora-Sinaloa lowlands (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Usinger 1944; Ryckman and Ryckman 1967; Ibarra-Cerdeña et al. 2009; Bern et al. 2011, 2020; Peterson et al. 2002).

The wild habitats and hosts of ***Triatoma barberi*** [Tc/H] remain unknown; its distribution, however, matches that of southern *Neotoma mexicana* populations between the Trans-Mexican Volcanic Belt and the Isthmus of Tehuantepec. In addition, non-wild *T. barberi* appear to preferentially feed on rodents including *Neotoma*, cotton and kangaroo rats, or spiny pocket, pigmy, deer, and brush mice; other reported blood sources were opossums or synanthropic mice and rats. *Triatoma barberi* lays adhesive eggs, which is suggestive of association with tree habitats. In the absence of direct evidence, we tentatively group this species with its woodrat-associated closest relatives; we, in any case, would not be surprised if it is eventually shown to associate primarily with arboreal rodent nests. All *T. cruzi* stocks from *T. barberi* we are aware of were isolated from non-wild bugs and belonged in DTU TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985; Carcavallo et al. 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Ibarra-Cerdeña et al. 2009; Peterson et al. 2002).

Triatoma rubida [Tc/L/H] has been reported from the nests of *N. albigula* and *N. lepida* and may also associate with *N. micropus* and *N. mexicana* in the Chihuahua Desert; the bugs may also feed on raccoons, and one population is associated with bat roosts (see Sect. 2.4). *Trypanosoma cruzi* stocks from *T. rubida* characterized to date were 99% TcI; TcIV was reported once. ***Triatoma neotomae*** [Tc] is only known from *N. micropus* nests in northeastern Mexico-southern Texas; Usinger (1944, p. 17) stressed that records of *T. neotomae* with *N. albigula* are incorrect – they refer to *T. rubida*. ***Triatoma nitida*** [Tc/H] from Mesoamerica is similar to *T. neotomae* and may also associate with *Neotoma* (likely *N. mexicana* and/or *Neotoma chrysomelas*) in the wild; the only record of wild *T. nitida* we are aware of, however, is from a cave in the pine-oak forests of central-western Guatemala. *Triatoma nitida* has been found infected with *T. cruzi* TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Brenière et al. 2016; Usinger 1944; Ryckman and Ryckman 1967; Ibarra-Cerdeña et al. 2009; Bern et al. 2011, 2020; Monroy et al. 2003a).

Four species in the *Triatoma phyllosoma* group (sensu Monteiro et al. 2018, yet including *Triatoma indictiva*) have been found in association with woodrat nests, but they appear to behave as generalist "stalkers" with a wider range of vertebrate hosts (see below and Sect. 3.1.2). ***Triatoma sanguisuga*** [Tc/L/H], however, displays a somewhat intermediate behavior: western populations (from the dry central-southern United States plain grasslands and the semiarid Tamaulipan mezquital) are fairly tightly associated with *Neotoma micropus* nests, whereas eastern populations (from the moister southeastern United States) infest *Neotoma floridana* nests but are also found in hollow or dead trees (with squirrels, raccoons, or opossums), root cavities (with armadillos), and *Sabal* palms (with tree frogs); both western and eastern *T. sanguisuga* may also occur with *Sigmodon* cotton rats and among rocks.

Triatoma indictiva [Tc/L/H] appears to be genetically very close to *T. sanguisuga* and has also been reported from woodrat nests and trees. About 74% (CI_{95} 68–80%) of *T. cruzi* stocks from *T. sanguisuga* ($n = 182$) and *T. indictiva* ($n = 30$) were TcIV; the rest were TcI (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Usinger 1944; Ibarra-Cerdeña et al. 2009; Bern et al. 2011, 2020; Mitchell 2013).

As mentioned above, four *Triatoma phyllosoma*-group putative "stalker" species may associate with woodrats but often feed on other hosts, mainly in rocky habitats (Sect. 3.1.2; Table 1): *Triatoma gerstaeckeri*, which occupies *N. micropus* nests; *Triatoma recurva*, which occasionally infests *Neotoma* nests; and the Mexican *Triatoma mazzottii* and *Triatoma phyllosoma*, which have been found associated with, respectively, *Neotoma* and *Hodomys* woodrats (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Usinger 1944; Ibarra-Cerdeña et al. 2009; Bern et al. 2011, 2020).

2.2.2 Other Mammal Ground Nests

In Cuba, ***Bolbodera scabrosa*** [−] occurs with largely ground-dwelling rodents known as hutias (*Capromys* and *Mesocapromys*), but whether this is a tight association remains unclear (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Usinger 1944). A further putative ground-nest specialist is ***Panstrongylus tupynambai*** [Tc/H] of the Uruguayan-southern Brazilian grasslands; this species occurs among rocks and stones, where it likely associates with rodent and marsupial nests but may also feed on birds or squamate reptiles (Georgieva et al. 2017; Patterson et al. 2009; Martins et al. 2006). *Panstrongylus howardi* has been found in peridomestic rodent nests built under brick or timber piles and among bromeliads (Villacís et al. 2015); as with most *Panstrongylus*, we suspect that in the wild it may primarily associate with arboreal rodent nests (see Table 1 and Sect. 2.3.2).

Triatoma rubrofasciata [Tc/L/H] is most likely native to India and was introduced into the Americas (and other places) via sea trade; this was possible because the bugs are tightly associated with the black-rat *Rattus* lineage including the "ship rats," among which *T. rubrofasciata* transmits *Trypanosoma conorhini* (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Justi et al. 2016).

Other triatomines (including host-generalist "stalkers") that may sporadically infest the nests of ground-dwelling rodents, particularly those built among rocks/stones, are listed in Table 1 (see also Sects. 2.1.1, 2.2.1, 3.1.2, and 3.2.2).

2.3 Arboreal Nests

2.3.1 Arboreal Bird Nests

The *Psammolestes* specialize in exploiting bird nests – and, in particular, ovenbird (Furnariidae) vegetative nests – in dry/open ecoregions of South America east of the Andes; the bugs may also feed on mice or opossums that take over the birds' nests. ***Psammolestes arthuri*** [Tc/H] is primarily associated with *Phacellodomus rufifrons/inornatus* ovenbirds in the Orinoco Llanos and adjacent semiarid and dry-forest ecoregions; it also occurs in nests of *Icterus* troupials; *Cacicus* caciques; *Psarocolius* oropendolas; and *Campylorhynchus*, *Troglodytes*, and *Pheugopedius* wrens. *Psammolestes arthuri* sometimes shares its nest microhabitats with other triatomines; in line with the idea that *Psammolestes* is a true bird-nest specialist, 99.3% (CI_{95} 98.9–99.5%) of 4180 bugs caught in 568 bird nests in northern Venezuela and the Colombian Llanos were *Ps. arthuri*; the putative host-generalist "stalkers" *R. prolixus* and *T. maculata* were found only sporadically. Most of the *T. cruzi* stocks from *Ps. arthuri* characterized to date were TcI, but TcIII has also been reported (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Abad-Franch et al. 2009; Di Iorio and Turienzo 2009; Cruz-Guzmán et al. 2014; Velásquez-Ortiz et al. 2019).

South of the Amazon basin, ***Psammolestes tertius*** [Tc/L/H] is common across the Brazilian Cerrado savanna and semiarid Caatinga; the ovenbirds *Phacellodomus ruber*, *Ph. rufifrons*, *Anumbius annumbi*, and *Pseudoseisura cristata* are *Ps. tertius*' main hosts, but nests of *Mimus* mockingbirds, *Piaya* squirrel cuckoos, and *Cacicus* may also be infested. In some cases, bird nests were occupied by *Didelphis* opossums or *Wiedomys* mice. Over half (52.7%, CI_{95} 50.1–55.3%) of 1318 ovenbird nests studied in Brazil were infested with *Ps. tertius* (10,078 bugs collected); the presence of the putative "stalkers" *Rhodnius neglectus* (10 nests, 104 bugs) and *Triatoma sordida* (14 nests, 213 bugs) appeared to be occasional, as is the finding of *Triatoma pseudomaculata* in ovenbird nests of the Caatinga. ***Psammolestes coreodes*** [–] occurs in dry forests and in grassland-savanna ecoregions of the southern and western Río de la Plata Basin, where it often occupies the nests of ovenbirds (*Ph. rufifrons*, *Pseudoseisura lophotes*, *Coryphistera alaudina*) and monk parakeets (*Myiopsitta monachus*, which do not breed in tree hollows as most parrots do, but in large, arboreal, often communal twig nests) (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Dias-Lima et al. 2003; Abad-Franch et al. 2009; Di Iorio and Turienzo 2009; Gurgel-Gonçalves and Cuba 2011; Emperaire and Romaña 2006; Carbajal-de-la-Fuente et al. 2008; Silva et al. 2018).

Southern *Psammolestes coreodes* populations share their microhabitats with two bird nest-specialized *Triatoma*. ***Triatoma platensis*** [Tc/H] appears to be primarily associated with ovenbird (*P. lophotes*, *A. annumbi*, *C. alaudina*) nests but can on occasion infest *Myiopsitta* nests. Conversely, ***Triatoma delpontei*** [Tc/H] is often found in nests of *M. monachus* in the Chaco, but only rarely in those of

Phacellodomus. *Triatoma platensis* and *T. delpontei* may associate with rodents (*Oligoryzomys*, *Graomys*) or marsupials (*Didelphis*, *Thylamys*, *Lutreolina*) that occupy the birds' nests, and *T. platensis* has been found with ground-dwelling *Microcavia* and in *Butia* palms. Chacoan "dark morph" populations of the closely related *Triatoma infestans* (Sect. 3.2.1), although not truly nest-specialized, have also been found in bird nests (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Abad-Franch et al. 2015; Di Iorio and Turienzo 2009; Noireau et al. 2000a; Martí et al. 2014). Other species that may use bird nests on occasion are listed in Table 1.

2.3.2 Arboreal Mammal Nests

Rhodnius paraensis [Tc] breeds inside the tree-hole nests of the white-faced spiny tree-rat, *Echimys chrysurus*, in eastern Amazonia; these nests can at times be also occupied by *Didelphis* opossums (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Miles et al. 1981). Although ***Rhodnius domesticus*** [Tc/H] from the Brazilian Atlantic Forest may occur in *Attalea* palms (Sect. 3.2.2), it has most often been reported from rodent (mainly *Phyllomys* spiny tree-rats) and opossum (*Didelphis*, *Marmosa*) nests built in terrestrial or epiphytic bromeliads, on trees, or in tree holes (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Leal et al. 1961). Similarly, southern populations of ***Rhodnius ecuadoriensis*** **genotype I** [Tc/L/H] (sensu Abad-Franch et al. 2009) seem to have shifted from palm to nest microhabitats. In the dry forests of central-western Ecuador, the bugs are found in palms and in tree nests of birds and mammals (opossums, squirrels, mice) (see Sect. 3.2.2), but in the seasonally dry Andean valleys of southern Ecuador, from where palms are absent, the bugs associate closely with nests of the tree squirrel, *Simosciurus stramineus/nebouxii*. These southern, squirrel-nest bug populations have been found infected with TcI – which was also detected in the squirrels (Barrett 1991; Abad-Franch et al. 2001, 2015; Cuba Cuba et al. 2002; Ocaña-Mayorga et al. 2018; Suárez-Dávalos et al. 2010). Wild ***Rhodnius ecuadoriensis*** **II** [Tc/H] (sensu Abad-Franch et al. 2009) have so far been reported only from a *Didelphis* nest inside a hollow tree of the northwestern Peruvian Andes (see Sect. 3.2.1). One mention of cacti may refer to bird or rodent nests built on or among cacti (Barrett 1991; Herrer et al. 1972). We finally note that several primarily palm-dwelling, host-generalist "stalker" *Rhodnius* species, including *Rhodnius prolixus*, *Rhodnius neglectus*, and *Rhodnius nasutus*, can occasionally infest bird or mammal nests built on trees or on palm crowns (see Sect. 3.2.2).

Panstrongylus megistus [Tc/L/H] is native to the moist Brazilian Atlantic Forest but extends into neighboring transitional ecoregions, into gallery forests of the Caatinga-Cerrado, and into the savanna/grassland ecoregions of the northern Río de la Plata Basin. It has been found in tree hollows, in clumps of epiphytic or terrestrial bromeliads, in entanglements of lianas, in false agaves, and among large fig-tree

roots – but, almost invariably, only within opossum, rodent, or (less often) bird nests built in such microhabitats. Wild bugs may occasionally feed on bats, agoutis, or armadillos. Adult *P. megistus* are seldom found inside the nests, suggesting that the bugs disperse soon after reaching adulthood. Most *T. cruzi* strains isolated from wild *P. megistus* and associated mammals have been characterized as TcI, but the bugs can also carry TcII or TcV/VI. *Panstrongylus megistus* may be involved in the transmission of *T. cruzi* TcI and TcII to the endangered *Leontopithecus* lion tamarins, which sleep in tree holes of the Brazilian Atlantic Forest (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Patterson et al. 2009; Leal et al. 1961; Forattini et al. 1970, 1977; Barretto 1979; Miles et al. 1982; Noireau et al. 2002a; Santos Jr et al. 2011, 2013; Lisboa et al. 2015; Kerr et al. 2016).

Panstrongylus lignarius [Tc/L/H] breeds inside tree-hole nests of *Echimys* spiny tree-rats, *Coendou* porcupines, *Didelphis* opossums, or *Tamandua* anteaters, as well as with *Potos* kinkajous and bats, in Amazonia; it has also been reported from palms (Table 2), including a *Mauritia carana* used by monkeys and the hollow trunk of a *Mauritia flexuosa* with nesting *Ramphastos* toucans. The Andean Marañón valley dry-forest population known as *Panstrongylus herreri* often infests guinea-pig pens and houses; in the wild, it may occupy tree-hole mammal nests too. Both *P. lignarius* from eastern Amazonia and *P. herreri* from the Marañón valley have been found infected with *T. cruzi* TcI and TcIV; TcII and TcIII also infect Andean bugs (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Izeta-Alberdi et al. 2016; Patterson et al. 2009; Miles et al. 1981; Lainson et al. 1979; D'Alessandro et al. 1981; Padilla et al. 2017). The closely related ***Panstrongylus humeralis*** [Tc/L/H] probably exploits similar tree-hole nest microhabitats in the moist forests of Central America and northern South America (Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Patterson et al. 2009). ***Panstrongylus rufotuberculatus*** [Tc/L/H] is widespread in (mainly) moist-forest Neotropical ecoregions, where it lives in tree hollows with kinkajous, opossums, or vampire bats; it may also feed on monkeys, was found once in a mouse nest on a *Phytelephas* palm, and can sporadically associate with armadillos (Tables 1 and 2). The few *T. cruzi* stocks from *P. rufotuberculatus* studied to date were all TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Patterson et al. 2009; Miles et al. 1981; Suárez-Dávalos et al. 2010; D'Alessandro et al. 1981).

The habitats and hosts of the small-bodied ***Panstrongylus mitarakaensis*** [Tc/L] remain unknown; it was collected at light on a granitic outcrop deep in the Guiana moist forests and does not seem to have been found elsewhere (Patterson et al. 2009; Bérenger and Blanchet 2007). ***Panstrongylus martinezorum*** [–/H] is only known from adult specimens found inside houses and public buildings of Puerto Ayacucho, Venezuela, in the transition between the Orinoco Llanos and the Guiana piedmont moist forests (Ayala 2009; Ayala et al. 2014).

Panstrongylus howardi [Tc/H] is endemic to the dry forests of coastal Ecuador, where it associates with peridomestic rodent nests in thick clumps of terrestrial bromeliads or under timber and brick piles; the bugs were reported to feed on *Proechimys* spiny rats and *Rattus* black rats. Wild *P. howardi* were found in a mouse nest on an *Aiphanes* palm (Patterson et al. 2009; Villacís et al. 2015; Abad-Franch et al. 2001; Suárez-Dávalos et al. 2010). In dry northwestern Peru, ***Panstrongylus chinai*** [Tc/L/H] breeds in adobe and wooden-walled thatch-roofed houses, in chicken coops, and under tree trunks, but will also colonize in peridomestic stone-wall goat enclosures (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Patterson et al. 2009; Abad-Franch et al. 2001; Cuba Cuba et al. 2002). ***Panstrongylus howardi*** and *P. chinai* are very closely related (Lent and Wygodzinsky 1979; Patterson et al. 2009; Barnabé et al. 2020); the sparse observations available make us suspect that, in the wild, both may be primarily associated with rodent nests built at the bases of hollow trees or among terrestrial plants (bromeliads, bushes) and perhaps rocks. A few *T. cruzi* samples taken from these sister species were all typed as TcI (Brenière et al. 2016; Izeta-Alberdi et al. 2016; Costales et al. 2015). ***Panstrongylus guentheri*** [Tc/L/H] is a further putative ground-level tree-nest specialist. It occurs in the savanna-grassland ecoregions of the Río de la Plata Basin, where it seems to associate with the nests of opossums, rodents, and perhaps birds, as well as with armadillos; *P. guentheri* has been reported from peridomestic timber piles. The habitats and hosts of ***Panstrongylus diasi*** [Tc/H] and ***Panstrongylus lenti*** [−/L/H] remain unknown (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Patterson et al. 2009). We recall that moist-forest populations of the widely spread *Panstrongylus geniculatus* (Sect. 2.1.1) often associate with rodent nests built inside fallen logs or among tree roots (Carcavallo et al. 1985, 1998; Barrett 1991; Patterson et al. 2009; Batista 2018; Batista et al. 2019).

Many species of Triatominae have been reported as associated with opossums (*Didelphis*, *Philander*, *Marmosa*, *Metachirus*, *Caluromys*, *Thylamys*, and *Lutreolina*), yet most of them are putative host-generalist "stalker" species that seem to infest opossum nests or "beds" opportunistically. Some *Panstrongylus*, *Rhodnius domesticus*, and perhaps *Rhodnius paraensis* are likely exceptions to this rule (see above), as are the two known species of *Microtriatoma*. ***Microtriatoma trinidadensis*** [Tc/L/H] is a widespread, moist forest-adapted, small, dark-brown, dorsoventrally flattened triatomine that lives between the dead/folded leaves with which opossums build their "beds" and some arboreal rodents line their tree-hole nests; it has also been found in epiphytic bromeliads, under loose tree bark, and, occasionally, in palms (Tables 1 and 2). The bugs feed on the nesting mammals and likely prey on nest-dwelling invertebrates; sloths and bats have also been reported as blood sources (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Georgieva et al. 2017; Abad-Franch et al. 2015; Miles et al. 1981; Gaunt and Miles 2000). The morphologically similar ***Microtriatoma borbai*** [Tc] associates with opossums and rodents in epiphytic bromeliads (and probably trees) of the Brazilian Atlantic Forest and has been found with *Didelphis* in *Mauritia*

palms of the Cerrado; in the laboratory, *M. borbai* did not feed on either mice or humans (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, al. 1998; Barrett 1991; Rodrigues et al. 1992; Gurgel-Gonçalves et al. 2012a). The two known *Parabelminus* species occur in the moist Brazilian Atlantic Forest, where both occupy microhabitats comparable to those of *Microtriatoma* – *Didelphis* nests on *Attalea* palms or tree holes (***Parabelminus carioca*** [Tc]) and epiphytic bromeliads with rodents, lizards, and tree frogs (*Parabelminus yurupucu*) (Lent and Wygodzinsky 1979; Abad-Franch et al. 2015; Carcavallo et al. 1985, 1998; Dujardin et al. 2002; Barrett 1991; Miles et al. 1982). It is unclear whether *Microtriatoma* and *Parabelminus* behave primarily as "sit-and-wait" or as "stalker" foragers (or both); we tentatively treat them as arboreal-nest specialists – except for *Pb. yurupucu*, which we (also tentatively) include in the epiphyte-associated "stalkers" (Sect. 3.2.3).

We note that a commonly reported bug-vertebrate association is that of palm-dwelling *Rhodnius* species with opossums (Gaunt and Miles 2000); other arboreal, likely opportunistic "stalker" triatomines that may associate with opossums, include species of *Belminus*, *Eratyrus*, and *Triatoma*. These instances will be reviewed in Sects. 3.1 and 3.2.

Although the primary hosts of *Triatoma lecticularia* and *Triatoma sanguisuga* are probably *Neotoma* woodrats (see Sect. 2.2.1), eastern populations of both species can occur in trees. *Triatoma sordida* and *Rhodnius stali* occasionally infest arboreal coati (*Nasua*) nests in the Pantanal wetlands (Santos et al. 2015, 2019) but are, respectively, primarily tree- and palm-dwelling – and also putative "stalkers" (see Sects. 3.2.1 and 3.2.2).

2.4 Bat Roosts

Cavernicola pilosa [Tc(*marinkellei*)/L/H] is tightly associated with bat roosts in caves and hollow trees. The bugs have been found with *Phyllostomus*, *Anoura*, *Carollia*, *Pteronotus*, *Desmodus*, *Noctilio*, *Eumops*, *Molossus*, *Saccopteryx*, and *Myotis* bats. *Cavernicola pilosa* may also occupy bat-inhabited palms and house-wall holes. We suggest that *C. pilosa* can be regarded as a "sit-and-wait" specialist exploiting the roosting sites (akin to "communal nests") of its preferential hosts. In caves, *C. pilosa* colonies tend to occupy the upper wall sections and the ceilings, where the bugs are physically close to roosting bats. ***Cavernicola lenti*** [Tc] is morphologically very similar to *C. pilosa* and likely also a bat-roost specialist; the only record we are aware of is from a large, live hollow tree of central-northern Amazonia with *Phyllostomus* leaf-nosed bats, *Rhipidomys* climbing rats, and *Eratyrus mucronatus*. Both *Cavernicola* species are often infected with the bat parasite *T. cruzi marinkellei* and probably transmit *T. cruzi* TcBat, which is common in bats but rarely infects *Rhodnius*, *Triatoma*, or *Panstrongylus*. *Cavernicola pilosa* is hard to rear in the laboratory unless fed on bats in a dark, moist environment; *C. lenti* readily feeds on mice (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011;

Atzingen et al. 2007; Oliveira et al. 2008; Pinto et al. 2015; Barrett and Arias 1985; Marcili et al. 2009).

Although *Triatoma rubida* appears to be primarily a "sit-and-wait" woodrat-nest specialist (Sect. 2.2.1), one population is associated with *Myotis* fish-eating bats roosting among rocks and on cliff crevices on the arid shores of the Gulf of California (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Georgieva et al. 2017). *Panstrongylus geniculatus* (Sect. 2.1.1) occurs in caves with roosting *Anoura*, *Carollia*, *Phyllostomus*, *Mormoops*, *Natalus*, and *Pteronotus* in Trinidad and in caves with the three latter genera plus *Leptonycteris* in coastal Venezuela; *Panstrongylus rufotuberculatus* (Sect. 2.3.2) may also associate with *Desmodus* roosts in hollow trees (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; D'Alessandro et al. 1981). Several further triatomine-bug species have been found in caves and large hollow trees, often in association with bats, but all appear to be primarily host-generalist "stalkers" (see Table 1 and Sects. 3.1.1 and 3.2.1).

About 79% (CI_{95} 72–85%) of *T. cruzi* strains isolated from bats in 15 genera have been identified as either TcBat (40%; 7 bat genera) or TcI (39%; 11 bat genera), but infections with TcII, TcIII, TcIV, and TcVI have also been reported (Brenière et al. 2016; Izeta-Alberdi et al. 2016).

3 "Stalker" Host Generalists

3.1 Terrestrial Microhabitats

3.1.1 Caves

Two species and one population of American triatomines have phenotypes that strongly suggest adaptation to, and hence true association with, cave microhabitats (see Taylor 2008). ***Triatoma mopan*** [Tc] is known only from caves of the eastern-most Petén-Veracruz moist forests in Belize; compared with its closest relatives in the *Triatoma dimidiata* complex (sensu Monteiro et al. 2018), *T. mopan* has smaller eyes and ocelli; a longer, more slender head; more and longer rostrum sensilla; and an overall lighter hue including light forewings. Other cave populations (from moist forests of northern Guatemala) in this species complex have also small eyes/ocelli and light-colored forewings but are genetically indistinguishable from ***Triatoma dimidiata* group I** [Tc] (sensu Monteiro et al. 2018; see below). In caves regularly visited by humans, *T. mopan* and cave *T. dimidiata* I may feed on bats (*Desmodus*, *Myotis*), opossums (*Didelphis*, *Philander*), synanthropic rodents (*Rattus*, *Mus*), birds, humans, and some domestic animals (Lent and Wygodzinsky 1979; Dorn et al. 2018; Bargues et al. 2008; Monteiro et al. 2018; Dorn et al. 2016; Stevens et al. 2014). ***Hermanlentia matsunoi*** [−] has only been collected (stalking humans) in caves of the upper Marañón valley dry forests in the northern Peruvian Andes; *H. matsunoi* also has a very long head, small eyes, and light-colored forewings

(Lent and Wygodzinsky 1979; Monteiro et al. 2018; Barrett 1991; Fernández-Loayza 1989). *Triatoma mopan*, cave *T. dimidiata* I, and *H. matsunoi* all behave as putative opportunistic "stalkers" that will try to feed upon the hosts that become available in their cave microhabitats.

Other American triatomines that have been reported from caves but may be primarily associated with non-cave microhabitats are listed in Table 1; *Triatoma confusa* from Cuba and *Triatoma obscura* from Jamaica may also occur in caves (see Sects. 3.1.2 and 3.2.1 and refs. Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Atzingen et al. 2007; Rojas et al. 1989; Villegas-García and Santillán-Alarcón 2001; Obara et al. 2012). As noted in Sect. 2.2.1, wild *Triatoma nitida* are known only from a cave (Monroy et al. 2003a).

3.1.2 Rocks and Stones

Dipetalogaster maxima [Tc/H] lives among exfoliating rocks in the xeric scrub and dry forests around the Sierra de la Laguna, Baja California, Mexico, where this bug is known as *chinche piedrera* – literally, "stone-dwelling bug." These large, bold triatomines actively attack their hosts (*Sauromalus* and *Petrosaurus* lizards, *Neotoma* woodrats, and, on occasion, humans) even in open daylight, and have been found infected with *T. cruzi* TcI (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Jiménez and Palacios 1999).

Most members of the *Triatoma phyllosoma* species group (sensu Monteiro et al. 2018, yet including *Triatoma indictiva*) are associated with rocky environments, but some have also been reported from other habitats including mammal nests and burrows, hollow trees, or palms (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002). Given the sparse observations, below we tentatively treat all *T. phyllosoma*-group species, save *Triatoma sanguisuga* and *T. indictiva* (see Sect. 2.2.1), as rock-/stone-dwelling host-generalist "stalkers" but will also emphasize several dubious instances.

Wild ***Triatoma gerstaeckeri*** [Tc/L/H] of the dry southeastern United States and northeastern Mexico often infest *Neotoma* woodrat nests but also occur with *Otospermophilus* rock squirrels (in caves and other rocky habitats) and in the shallow burrows of *Sylvilagus* desert cottontails, as well as under shrubs and cacti with mice or opossums. Wild *T. gerstaeckeri* may also feed on porcupines, raccoons, weasels, foxes, armadillos, deer, vultures, crows, snakes, or crickets. These observations suggest that *T. gerstaeckeri* behaves as a typical opportunistic "stalker," but whether it is primarily associated with rocks/stones or with terrestrial plant microhabitats (Sect. 3.1.3) remains unclear. Under experimental conditions, Mexican bugs selected dirt or concrete surfaces (at or close to ground level) for resting; in the same area, wild populations infest *Neotoma micropus* nests. In woodland or *Opuntia*-cactus patches at San Antonio, Texas, the bugs were common near (but rarely inside) *N. micropus* nests built in/under dead logs and yuccas or at the base of cacti; in coastal southern Texas, *T. gerstaeckeri* were breeding under cement slabs

in a patio, and in southern-central Texas adult bugs including newly molted specimens were said to hide under (presumably wooden) siding of external house walls. About 61% (CI_{95} 57–64%) of 655 *T. cruzi* stocks isolated from this species were TcI, and 39% (CI_{95} 36–43%) were TcIV (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Georgieva et al. 2017; Brenière et al. 2016; Bern et al. 2020; Wood and Wood 1961; Pippin 1970; McPhatter et al. 2012; Torres-Estrada et al. 2002; Beard et al. 2003; Molina-Garza et al. 2007; Wozniak et al. 2015). We found no information on the wild habitats and hosts of the closely related ***Triatoma mexicana*** [Tc/L/H] from the eastern Trans-Mexican Volcanic Belt; in northeastern Guanajuato, it breeds under/among stones used to build peridomestic fences (Salazar-Schettino et al. 2007, 2010).

There seems to be no records about the ecology of wild ***Triatoma dimidiata*** **group II** [Tc/L/H] (sensu Monteiro et al. 2018) – the sub-clade of the Mexican Gulf coast and southeastern Sierra Madre slopes known for some time as *T. dimidiata maculipennis*. Synanthropic populations appear to be common in stone- or mud-walled houses and in peridomestic stone walls. In Veracruz, invasion of houses by adult *T. dimidiata* II is more frequent in communities on the Sierra Madre slopes than in nearby, lowland communities (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Salazar-Schettino et al. 2010; Ramos-Ligonio et al. 2010; Torres-Montero et al. 2012). Populations of ***Triatoma dimidiata*** **group I** [Tc/L/H] (sensu Monteiro et al. 2018, although some records from the moist forests of northern Guatemala may refer to *Triatoma huehuetenanguensis*) occupy rocky habitats including rock piles, cliff crevices, stone walls, and archeological remains – a stony habitat where ***Triatoma hegneri*** [Tc/L/H] has also been recorded. Wild *T. dimidiata* (I/II) also occur on trees and palms and may associate with a highly diverse fauna including primates (*Cebus*), rodents (*Coendou*, *Oryzomys*, *Peromyscus*, *Sigmodon*), opossums (*Didelphis*, *Philander*), bats (*Desmodus*, *Myotis*), carnivores (*Urocyon*), armadillos, birds (hawks, herons, parrots, doves), snakes, lizards, geckos, or toads. Most *T. cruzi* samples from *T. dimidiata* (I/II), and all samples from bugs caught in the wild, were TcI, but infections with DTUs TcII to TcVI have also been reported (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2015; Mazzotti 1943; Monroy et al. 2003b; Parra-Henao et al. 2016; Ramos-Ligonio et al. 2012; Pech-May et al. 2019). Recent data suggest that adult, wild ***Triatoma huehuetenanguensis*** [Tc/L/H] from northwestern Yucatán may feed on tree-dwelling squirrels (*Sciurus*), porcupines (*Coendou*), kinkajous (*Potos*), and doves (*Zenaida*), although deer, humans, domestic animals (dogs, cows, pigs, chickens, turkeys), house mice, and frogs were also fed upon. We tentatively include this species in the group of rock-dwelling, host-generalist "stalkers." *Triatoma huehuetenanguensis* carry *T. cruzi* TcI, TcII, TcVI, and likely also TcV (Pech-May et al. 2019; Dumonteil et al. 2018; Moo-Millan et al. 2019).

Triatoma recurva [Tc/L] of the Sonora-Mojave and northwestern Chihuahua deserts appears to infest *Neotoma* nests only occasionally and feeds also on squamate reptiles, rock squirrels, and opossums. At the western end of the Trans-Mexican

Volcanic Belt, the closely related ***Triatoma longipennis*** [Tc/H] was found to be common in stony habitats occupied by *Sigmodon* cotton rats, *Reithrodontomys* harvest mice, and other small rodents (*Baiomys*, *Liomys*); the bugs (which often carried *T. cruzi* TcI and rarely TcIII/TcIV) were however feeding on a wide variety of hosts including rodents but also raccoons, armadillos, opossums, skunks, lizards, felids, or shrews. There are records of *T. longipennis* from caves (with *Artibeus* fruit bats), cliffs, and hollow trees/cacti. The sympatric ***Triatoma picturata*** [Tc/H] occurs in stone piles/walls, cliffs, caves, ground mammal burrows, and occasionally hollow trees/cacti; it has been shown to carry TcI. ***Triatoma pallidipennis*** [Tc/H] infests the terrestrial nests of *Hodomys* woodrats on the southwestern slopes and valleys of the Sierra Madre; it can also feed on *Sigmodon* cotton rats, *Peromyscus* brush mice, *Baiomys* pigmy mice, and other hosts including armadillos, opossums, or cave-roosting bats. Infections with TcI and, more rarely, TcII to TcIV have been reported in synanthropic bugs. The closely related, sympatric ***Triatoma bassolsae*** [Tc/H] is only known from domestic-peridomestic habitats. In southwestern Mexico (west of the Isthmus of Tehuantepec), ***Triatoma mazzottii*** [Tc/H] has been found associated with *Neotoma* but also occurs in caves and rocky habitats; similarly, ***Triatoma phyllosoma*** [Tc/H] may associate with *Hodomys* woodrats, ground squirrels, pigmy mice, skunks, or lizards; one domestic bug carried *T. cruzi* TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Rojas et al. 1989; Villegas-García and Santillán-Alarcón 2001; Ryckman et al. 1965; Bosseno et al. 2002; Martínez-Ibarra et al. 2008; Bosseno et al. 2009; Gorchakov et al. 2016; López-Vivas et al. 2018). We found no data on ***Triatoma brailovskyi*** [−/L] or ***Triatoma gomeznunezi*** [−/L], which most likely belong in this group (Monteiro et al. 2018; Carcavallo et al. 1998).

Mepraia gajardoi [Tc/H] and ***Mepraia parapatrica*** [Tc] occupy the rocky seashore (including some small islands close to the coast) of, respectively, the hyperarid Atacama Desert and the Atacama-Matorral transition in northern Chile; they associate with birds (penguins, seagulls, pelicans, and vultures), mammals (sea lions, sea otters, rodents, and free-ranging goats), lizards, and snakes. *Mepraia gajardoi* males have short wings, whereas *M. parapatrica* males have either short or long wings; females of all known *Mepraia* are wingless. Wild *M. gajardoi* have been found infected with *T. cruzi* TcII at a fairly higher frequency (~45%; CI_{95} 34–57%) than with TcI, TcV, or TcVI (all ~18%); TcII, TcV, and TcVI have been found in *M. parapatrica* island populations (Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Brenière et al. 2016; Frías-Lasserre 2010; Rives-Blanchard et al. 2017). ***Mepraia spinolai*** [Tc/H] (whose males can be long-winged, short-winged, or wingless) occurs in rocky habitats of the less hostile dry Chilean Matorral, where it is mainly associated with rodents (*Abrocoma*, *Lagidium*, *Oligoryzomys*, *Phyllotis*, *Octodon*, or *Abrothrix*) and introduced European rabbits; *Thylamys* mouse opossums, lizards, goats, canids, felids, and humans may also be fed upon by these bugs, which have not been reported to feed on birds. Most *T. cruzi* from *M. spinolai* have been characterized as TcI (65%; CI_{95} 59–72%) or TcII (19%; CI_{95} 15–25%), with TcV and TcVI both at

frequencies <9%. The same parasite DTUs infected four major wild hosts of rock-dwelling *M. spinolai* (*Abrothrix*, *Octodon*, *Phyllotis*, and *Thylamys*) at one wildlife reserve in the core Chilean Matorral, where frequencies varied modestly from 30% (CI_{95} 24–40%) for both TcI and TcII to 17% (CI_{95} 11–25%) for TcVI; all four DTUs were also isolated from feral goats in the same area. A recent study characterized *T. cruzi* from *Octodon*, *Oligoryzomys*, *Phyllotis*, and *Thylamys* sharing stony habitats (some including *Puya* terrestrial bromeliads) with *M. spinolai* in the same DTUs and at similar frequencies (30–40% for TcI and TcII, 15–16% for TcV and TcVI); *Abrothrix* and *Abrocoma* were also infected, but DTUs could not be determined (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Frías-Lasserre 2010; Molina et al. 2004; Rozas et al. 2007; Bacigalupo et al. 2010; Oda et al. 2014; Chacón et al. 2016; Ihle-Soto et al. 2019).

Within the *Mepraia* species group (sensu Monteiro et al. 2018), the normal-winged ***Triatoma eratyrusiformis*** [Tc/L/H] and ***Triatoma breyeri*** [Tc/L/H] are associated with inland stony habitats occupied by rodents (especially *Microcavia* mountain cavies) in the dry-to-semiarid ecoregions of the eastern side of the southern Bolivian and northern-central Argentinean Andes. *Triatoma eratyrusiformis* is a bold species that also lives with cavies in peridomestic shrub fences, where the bugs appear to carry mainly *T. cruzi* TcI but may also transmit TcVI and perhaps TcII or TcV. *Triatoma ninioi*, which was synonymized with *T. eratyrusiformis*, preferentially lives in moister gallery forests, and a population of bugs superficially similar to *T. breyeri* occurs in dry montane forests of the southeastern Bolivian Andes (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Cécere et al. 2016; Cavallo et al. 2016).

Triatoma vitticeps [Tc/L/H] occupies stony habitats along the moist Atlantic Forest ranges of southeastern Brazil, where it associates with *Kerodon* rock cavies but may also feed on opossums, armadillos, birds, or lizards. The positive correlation of house invasion by *T. vitticeps* with both steeper terrain and standing-forest patches has been taken to suggest that wild bug populations are particularly common on stony hill-slopes where anthropogenic landscape disturbance is less extensive. In line with this view, no *T. vitticeps* (or any other triatomines) were found in nearly 400 *non*-stony microhabitats sampled in a hilly Atlantic Forest area where 450+ *T. vitticeps* were caught invading houses over a 7-year period. *Triatoma vitticeps* are often infected with *T. cruzi*, with a reported predominance of TcII (83%, CI_{95} 71–90%); TcI, TcIII, and TcIV can infect this species too. The closely related *Triatoma melanocephala* might also use wild rocky habitats in some parts of its range but has so far been recorded only from terrestrial bromeliads (Sect. 3.1.3) (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Gonçalves et al. 1998; Leite et al. 2011; Brito et al. 2017a; Dario et al. 2018).

Andean populations of ***Triatoma infestans*** [Tc/L/H] are common in wild rocky habitats of the Bolivian dry montane forests and parts of the Central Andean Puna. The bugs often associate with rodents, including cavies (*Galea*), vizcachas

(*Lagidium*), mountain degus (*Octodontomys*), and the smaller grass mice (*Akodon*), bolo mice (*Necromys*), and leaf-eared mice (*Phillotys* and *Graomys*); mouse opossums (*Thylamys*) also use rocky shelters shared with wild *T. infestans*. Although domestic *T. infestans* can carry any DTU, almost all *T. cruzi* stocks from wild Andean *T. infestans* typed so far belong to TcI (99%, CI_{95} 97.2–99.7%), with a small minority classified as TcIII. TcI was also the only *T. cruzi* strain found in a sample of wild rodents (*Phyllotis*, *Necromys*, and *Akodon*; $n = 31$) and mouse opossums (*Thylamys*; $n = 15$) associated with rock-dwelling *T. infestans* in the Bolivian Andes (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Noireau et al. 2000a; Noireau et al. 2000b, 2005a; Rojas Cortez et al. 2006; Rojas Cortez et al. 2007; Buitrago et al. 2010; Brenière et al. 2017). Wild *T. infestans* populations of the dry Chaco are primarily arboreal (Sect. 3.2.1).

The seven species in the *Triatoma brasiliensis* complex (sensu Monteiro et al. 2018) of the semiarid Brazilian northeast are all rock-dwelling. Wild ***Triatoma brasiliensis*** [Tc/L/H] seem to be primarily associated with rocks and rock-dwelling *Kerodon* rock cavies, *Galea* cavies, and *Thrichomys* punares, although blood meals from *Trinomys* spiny rats, *Wiedomys* mice, and *Oecomys* rice rats have also been reported. Other than rodents, wild rock-dwelling *T. brasiliensis* may feed upon opossums (*Didelphis*, *Monodelphis*), armadillos, skunks (*Conepatus*), bats (*Phyllostomus*), birds, lizards (*Tropidurus*, *Tupinambis*, *Mabuya*, and *Phyllopezus*), amphibians (*Proceratophrys*), arthropods, and free-ranging domestic animals (dogs, goats, sheep, cows, or pigs); hungry *T. brasiliensis* will also readily stalk humans in the bugs' rocky habitats. Some populations from rock-free subregions have adapted to shrubby cacti (Sect. 3.1.3). Infections with *T. cruzi* TcI, TcII, and TcIII have been reported in wild, rock-dwelling *T. brasiliensis*; the number of samples studied to date is however too small to draw conclusions about possible DTU associations. Other members of the complex (***Triatoma juazeirensis*** [Tc/L/H], ***Triatoma sherlocki*** [Tc/H], ***Triatoma lenti*** [Tc/H], ***Triatoma bahiensis*** [−/H], ***Triatoma petrocchiae*** [Tc/H], and ***Triatoma melanica*** [Tc/H]) have also been consistently reported from rocky habitats of the semiarid Caatinga and, especially for *T. melanica*, the Cerrado. In line with the reports on *T. brasiliensis* summarized above, these bugs appear to associate mainly with mammals (rodents, armadillos, opossums) and lizards, yet birds, humans, and domestic animals (goats, horses) have also been identified as possible blood sources (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Carbajal-de-la-Fuente et al. 2008; Noireau et al. 2002a; Costa et al. 1998, 2014; Ribeiro et al. 2019; Carbajal-de-la-Fuente et al. 2007; Almeida et al. 2009, 2016; Câmara et al. 2013; Souza et al. 2015; Sarquis et al. 2010; Bezerra et al. 2014, 2018; Mendonça et al. 2015; Lima-Oliveira et al. 2020).

Triatoma arthurneivai [−/L/H] and ***Triatoma wygodzinskyi*** [Tc/H] are rock-dwelling members of the *Triatoma pseudomaculata* species complex (sensu Monteiro et al. 2018); both appear to associate with *Tropidurus* lizards, rodents, and

probably also birds and marsupials. Four further species whose natural habitats and hosts remain largely unknown belong in this complex: ***Triatoma guazu*** [Tc/H], which appears to occupy rocky habitats, likely in association with rodents, birds, lizards, and bats; ***Triatoma baratai*** [−/H], which was collected near a cave in the southwestern Cerrado; ***Triatoma williami*** [Tc/H], which may feed on rodents and skunks and was once caught stalking people during the day at a rocky-ground gallery-forest site of the northwestern Cerrado (RN Brito, pers. comm.); and ***Triatoma deaneorum*** [−/H], about which we found no information (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rodrigues et al. 1992; Obara et al. 2012; Noireau et al. 2002b; Carcavallo and Jurberg 2000).

Other rock-dwelling species that are likely close kin to *T. pseudomaculata* and allies are ***Triatoma costalimai*** [Tc/H] and its sister species, ***Triatoma jatai*** [−/H] (Monteiro et al. 2018). *Triatoma costalimai* lives in limestone outcrops of the central-northern Cerrado in association with rodents (*Kerodon* rock cavies plus probably *Thrichomys* and *Calomys*) and *Tropidurus* lizards, although it may also feed on birds, marsupials, or armadillos. Wild *T. jatai* use the same habitat, and probably associate with similar hosts, as *T. costalimai*. Infection of *T. costalimai* with *T. cruzi* is common; 21 stocks from wild-caught bugs were all TcI (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Lorosa et al. 1999; Machiner et al. 2012; Brito et al. 2017b; Teves et al. 2019; Gonçalves et al. 2013).

Most species in the *Triatoma rubrovaria* complex (sensu Monteiro et al. 2018) are primarily associated with rock-outcrop and stony-ground habitats in the Uruguayan savanna grasslands, where they appear to feed mainly on lizards and geckos and on other arthropods (e.g., cockroaches), although rodents (including *Cavia*), armadillos (*Dasypus*), opossums, goats, horses, cows, dogs, birds, and amphibians may also be fed upon by the bugs. ***Triatoma rubrovaria*** [Tc/H], ***Triatoma circummaculata*** [Tc/H], ***Triatoma carcavalloi*** [Tc/H], and ***Triatoma pintodiasi*** [−/H] match this profile. The few *T. cruzi* stocks characterized so far from bugs in this group were all TcIII found in *T. rubrovaria* (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Martins et al. 2006; Jurberg et al. 2013; Lima 2017). ***Triatoma oliveirai*** [−], known only from a small area at the northeastern end of the Uruguayan savanna, associates with *Cavia* and was also found in rocks with deep crevices and cavities on a forested hill-slope (Lent and Wygodzinsky 1979; Carcavallo et al. 1985; 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Barcellos and Grazia 1989). Similarly, ***Triatoma klugi*** [−] is only known from the basalt-rock cliffs of Mount Malakov at the southern end of the Alto Paraná Atlantic Forest, where it may feed on rodents, birds, and cockroaches (Noireau et al. 2002a; Carcavallo et al. 2001; Emmanuelle-Machado et al. 2002). Three *Triatoma rubrovaria* complex species occur west of the Uruguay River. ***Triatoma limai*** [−/L] from the dry Chaco and Espinal is found under rocks, where it seems to associate with rodents. The more widely distributed ***Triatoma patagonica*** [Tc/L/H] occurs in rocky-ground habitats too but has also been reported

from rodent burrows, in/under fallen tree trunks, or under stones located inside caves; in the wild, *T. patagonica* may associate with cavies (*Microcavia*), leaf-eared mice (*Graomys*), and armadillos. Wild *T. patagonica* can be fairly bold and readily attack humans sleeping outdoors (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Mazza 1949; Ábalos and Wygodzinsky 1951; Wisnivesky-Colli et al. 2003). *Triatoma guasayana* is the only member of the *Triatoma rubrovaria* complex that does not seem to be primarily associated with rocky habitats; although it occurs under rocks (with rodents and toads) and under fallen tree trunks (with geckos), it most often occupies arboreal microhabitats (Sect. 3.2.1).

Triatoma jurbergi [Tc/H] and ***Triatoma vandae*** [Tc/L/H] of the *Triatoma sordida* complex (sensu Monteiro et al. 2018) have been found in rocky cliffs of the Cerrado, where the bugs were reported to feed on rodents, opossums, skunks, birds, and lizards; it seems likely that the closely related, sympatric, and morphologically very similar ***Triatoma matogrossensis*** [−/H] also uses rocky habitats, but we found no actual records (Georgieva et al. 2017; Jurberg et al. 2002; Carcavallo et al. 2002; Lorosa et al. 2003). Although *Triatoma sordida* is primarily arboreal (Sect. 3.2.1), wild Andean populations can occupy stony microhabitats, and the species has also been collected from stone walls (Noireau et al. 2005b; Gorla and Noireau 2010).

Triatoma boliviana [−/H] of the Central Andean Puna and northern Bolivian montane dry forests seems to be the only species in the *Triatoma dispar* lineage (sensu Monteiro et al. 2018) that is associated with stony microhabitats, where it likely feeds on rodents and on arthropods. One record suggests, however, that high-altitude populations of *Triatoma carrioni* (which closely resembles *T. boliviana*) may also exploit stony habitats in Peru and Ecuador (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Georgieva et al. 2017; Abad-Franch et al. 2001; Cuba Cuba et al. 2002; Martínez et al. 2007; Durán et al. 2014, 2016; Herrer 1955). Otherwise, the species in this lineage are primarily arboreal (Sect. 3.2.1).

The little-known Antillean *Triatoma* species (***Triatoma flavida/bruneri*** [−/H], ***Triatoma confusa*** [−], and ***Triatoma obscura*** [−/H]) appear to largely behave as "stalkers" in stony and cave habitats, where they may associate with hutias – *Capromys* and *Mesocapromys* in Cuba and *Geocapromys* in Jamaica (Lent and Wygodzinsky 1979; Carcavallo et al. 1985; Barrett 1991; Fraga et al. 2011). *Eratyrus cuspidatus* (Sect. 3.2.1) may also occur among rocks/stones. Finally, *Panstrongylus tupynambai* and *Panstrongylus chinai* may be found in stony habitats, but here we treat them as putative "sit-and-wait" nest specialists (see Sects. 2.2.2 and 2.3.2).

3.1.3 Terrestrial Plant Microhabitats

Several "stalker" triatomine-bug species have been recorded from dead logs and shrubby plants (bushes, terrestrial bromeliads, yuccas, agaves, or cacti) or among tree roots; none of them, however, seem to be primarily associated with such microhabitats, save two exceptions (Table 1). First, some populations of the primarily

rock-dwelling ***Triatoma brasiliensis*** [Tc/L/H] (Sect. 3.1.2) have adapted to live among the creeping stems and branches of *Pilosocereus* shrubby cacti in sedimentary, rock-free lowlands of the semiarid Caatinga; these cactus-dwelling bugs appear to be associated with *Galea* cavies (Ribeiro et al. 2019; Valença-Barbosa et al. 2014). A second exception might be ***Triatoma melanocephala*** [Tc/H], which in the wild is only known from terrestrial bromeliads of the dry-to-semiarid northeastern Brazil, where the bugs may associate with *Didelphis* and transmit *T. cruzi* TcI and perhaps TcIII (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Barrett et al. 1980; Sherlock and Guitton 1980; Rimoldi et al. 2012). *Triatoma melanocephala* could also occur in rocky habitats similar to those used by the closely related, largely parapatric *Triatoma vitticeps* (Sect. 3.1.2).

A few rock-dwelling "stalkers" also use terrestrial plant microhabitats on occasion. *Triatoma gerstaeckeri* (Sect. 3.1.2) may occur in bush and cacti with woodrats, mice, or opossums. Although no direct evidence seems to be available, it has been suggested that *Triatoma phyllosoma* might associate with the blue agave (*Agave tequilana*) in Oaxaca, Mexico (Ramsey and Schofield 2003). *Mepraia spinolai* and seemingly wild *Triatoma infestans* may occur under *Puya* terrestrial bromeliads in rocky habitats of the dry Chilean Matorral (Bacigalupo et al. 2006, 2010) and *Triatoma eratyrusiformis* under shrub fences in association with cavies (Cécere et al. 2016). Finally, some *Triatoma patagonica* live within fallen logs with ground-dwelling rodents (Sect. 3.1.2).

Terrestrial bromeliads and fallen logs provide habitat to wild *Triatoma guasayana*, *T. sordida*, and "dark morph" *T. infestans* (Noireau et al. 1999, 2000a, b, 2002a); these bugs, however, seem to be primarily tree-dwelling – as are *Triatoma maculata* and *Triatoma pseudomaculata*, which may associate with lizards and rodents in dead logs (see Sect. 3.2.1).

Most putative "sit-and-wait" woodrat-nest specialist triatomines can be found in terrestrial plant microhabitats (e.g., under bushes or cacti and in hollow logs) when their hosts nest there; eastern *Triatoma sanguisuga* populations may also use root cavities in association with armadillos (see Sect. 2.2.1). *Bolbodera scabrosa* has also been reported from fallen logs with *Capromys* hutias (see Sect. 2.2.2). Similarly, the putative arboreal-nest specialist *Panstrongylus megistus* may occur with rodents and opossums in clumps of terrestrial bromeliads (*Bromelia* spp., where *Rhodnius domesticus* has been found too) and false agaves (*Furcraea*), as well as among the complex roots of large fig trees (see Sect. 2.3.2).

3.2 Arboreal Microhabitats

3.2.1 Trees

Although little is known about their wild habitats and hosts, the species in the *Triatoma dispar* lineage (Monteiro et al. 2018), perhaps with the exception of *T. boliviana* (Sect. 3.1.2), seem to be primarily tree-dwelling (Lent and Wygodzinsky

1979; Carcavallo et al. 1985, 1998; Barrett 1991). ***Triatoma dispar*** [Tc/L/H] occurs in moist forests of southern Mesoamerica and the northern Andes down to Ecuador; it has been found to be associated with *Choloepus* sloths (which rest on tree forks or entangled lianas up in the canopy) but might also feed on kinkajous and monkeys. *Triatoma dispar* was reported attacking humans in a forest-canopy platform in Panama and infesting a barn with roosting bats (*Molossus* and *Myotis*, one of which carried *T. cruzi marinkellei*) in southern Ecuador (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Pinto et al. 2015). ***Triatoma nigromaculata*** [Tc/L/H] occurs in tree holes with opossums and birds and occasionally associates with armadillos, in the dry and montane ecoregions of northern Venezuela; it is often infected with *T. cruzi*, and house-invading adults carried TcI (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Carrasco et al. 2014). The habitats and hosts of wild ***Triatoma venosa*** [Tc/H] remain unknown, but morphological and biogeographic-ecological similarities with *T. dispar* (Lent and Wygodzinsky 1979; Monteiro et al. 2018) suggest that it is probably an arboreal species; bugs from Colombia carry *T. cruzi* TcI, TcIII, and TcIV (Guhl and Ramírez 2013; Herrera et al. 2007). Northern ***Triatoma carrioni*** [Tc/L/H] populations occur in montane cloud forests of central-western Ecuador, where they most likely live on trees and epiphytic bromeliads. In the drier inter-Andean valleys of southern Ecuador and northern Peru, *T. carrioni* occurs in tree holes with rodents and perhaps other mammals. One record from a cave and the association of at least some *Triatoma boliviana* populations with stony microhabitats (Sect. 3.1.2) suggest that southern *T. carrioni* from non-forested, high-altitude ecoregions may occur among stones too; in man-made habitats, these bugs have been shown to carry *T. cruzi* TcI (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Georgieva et al. 2017; Abad-Franch et al. 2001; Cuba Cuba et al. 2002; Herrer 1955).

Triatoma ryckmani [−/H] has been found under the bark of a large guanacaste tree (*Enterolobium*) in dry forest of northwestern Costa Rica. In the semiarid Motagua valley, Guatemala, *T. ryckmani* breeds inside dead/decaying portions of columnar *Stenocereus* cacti, apparently in association with rodents, as well as in clumps of *Tillandsia* epiphytic bromeliads growing on cacti (*Pereskia* and *Stenocereus*) and on trees (*Guaiacum*). *Tillandsia*-dwelling *T. ryckmani* were likely feeding on rodents, lizards, birds, and other arthropods. ***Triatoma bolivari*** [Tc/L] appears to be sister to the primarily arboreal *T. ryckmani*; it has been caught at light in preserved dry forests of coastal Jalisco, Mexico, where the bugs appeared to feed on *Sciurus* tree squirrels and birds and carried *T. cruzi* TcI plus, less frequently, TcII and TcIV (Georgieva et al. 2017; Sherlock and Morera 1988; Marroquín et al. 2004; Zeledón et al. 2010; Espinoza et al. 2013; Nguyen 2016).

Triatoma maculata [Tc/L/H] occurs in dry-to-semiarid ecoregions from northern Colombia to the savannas of Roraima in northern Brazil. In the wild, *T. maculata* lives under tree bark and in bird nests but has also been found in dead logs, palms, and epiphytic bromeliads; one population was associated with bats within the dry branches of *Stenocereus* columnar cacti in the northern Venezuelan dry forests. *Triatoma maculata* seems to prefer bird blood but may also feed on opossums,

rodents, anteaters, bats, squamate reptiles, or amphibians; all *T. cruzi* stocks from *T. maculata* characterized so far were TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Briceño et al. 2014).

In the Caatinga and the Cerrado, ***Triatoma pseudomaculata*** [Tc/L/H] is found in tree holes, under tree bark, in dead logs, and in arboreal termite nests; it may also infest ovenbird nests built in *Cereus* arboreal cacti and has occasionally been found in palms. Wild bugs associate with rodents, birds, lizards, and arthropods. The few *T. cruzi* stocks typed to date were TcI from domestic bugs (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Dias-Lima et al. 2003; Emperaire and Romaña 2006; Carbajal-de-la-Fuente et al. 2007, 2008; Venâncio 2010). We note that, in spite of their striking morphological and ecological-behavioral similarities (Lent and Wygodzinsky 1979), *T. maculata* and *T. pseudomaculata* appear to be only distant relatives (Monteiro et al. 2018).

The widespread ***Triatoma sordida*** [Tc/L/H] is common in tree holes, under tree bark, among tree roots, in fallen trees and cacti, in bird nests (on trees or arboreal cacti), and in epiphytic bromeliads of the dry-to-semiarid, open ecoregions of central South America; it has also been found in clumps of terrestrial plants (bromeliads, agaves, grasses), in palm crowns, and in *Galea* cavy burrows. Although often associated with birds (*Phacellodomus*, *Pseudoseisura*, and *Anumbius* ovenbirds; *Campylorhynchus* wrens; *Myiopsitta* and *Thectocercus* parakeets; *Turdus* thrushes; or *Guira* cuckoos), wild *T. sordida* may also feed on opossums (*Didelphis*, *Lutreolina*), rodents (*Akodon*, *Cavia*, *Galea*), monkeys (*Cebus*), bats, or squamate reptiles. *Triatoma sordida* often carries *T. cruzi* TcI but has also been found infected with TcII, TcV, TcVI, and TcBat; it is a candidate vector of *T. cruzi* to *Alouatta* howler monkeys in the humid Chaco and Paraná flooded savanna (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Di Iorio and Turienzo 2009; Noireau et al. 2000a, 2002a; Rolón et al. 2011; Martínez et al. 2016). ***Triatoma garciabesi*** [Tc/L/H] from the core dry Chaco is sister and very similar to, albeit often smaller and darker than, *T. sordida*; it is common in bird (mainly *Myiopsitta*, but also *Phacellodomus*) or rodent nests, in hollow trees, and under loose tree bark and has occasionally been found in epiphytic and terrestrial bromeliads (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1998; Di Iorio and Turienzo 2009; Noireau et al. 2000a; Jurberg et al. 1998; Canale et al. 2000).

Wild populations of ***Triatoma infestans*** [Tc/L/H] (see also Sect. 3.1.2) are widespread across the dry Chaco; these usually melanic bugs have been found most often inside hollow trees but also under tree bark, in dead tree and cactus trunks and branches, and, less frequently, in epiphytic and terrestrial bromeliads. They also infest the nests of ovenbirds (*Coryphistera* and *Phacellodomus*), parrots (*Myiopsitta* and the tree hole nesting *Thectocercus* and *Amazona*), antbirds (*Taraba*), and rodents on (or, more often, within) those tree microhabitats. Wild Chacoan

T. infestans have been shown to carry *T. cruzi* TcI and TcII (Di Iorio and Turienzo 2009; Noireau et al. 2000a; Martí et al. 2014; Noireau et al. 2000b, 2002a, 2005a; Brenière et al. 2017; Rolón et al. 2011; Noireau and Flores 1997; Ceballos et al. 2009, 2011; Waleckx et al. 2012).

Triatoma guasayana [Tc/L/H] belongs in the *Triatoma rubrovaria* group of primarily rock-dwelling species and occurs across the dry Chaco (Monteiro et al. 2018). Unlike its close relatives, *T. guasayana* has most often been reported as associated with arboreal habitats including tree and cactus trunks, tree holes, tree stumps, bird nests, and loose tree bark; it may also occur in bromeliads (terrestrial and epiphytic) and under logs or rocks. These bold bugs associate with birds, opossums, geckos, and toads and have been found infected with *T. cruzi* TcIII (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Di Iorio and Turienzo 2009; Noireau et al. 2000a, 2002a; Noireau and Flores 1997; Ceballos et al. 2009, 2011; Acosta et al. 2017).

Eratyrus mucronatus [Tc/L/H] is widespread across the Amazon moist forests and most adjacent ecoregions east of the Andes, including savannas, dry forests, montane forests, and coastal restingas. In Amazonia, this species occurs inside living hollow trees, where it associates with porcupines and other rodents, kinkajous, opossums, bats, and perhaps sloths and monkeys up in the canopy. Nymphs may feed on arthropods (*Amblypygi* whip spiders, *Nasutitermes* termites, or *Blaberus* cockroaches), and frogs and geckos have also been found inside *E. mucronatus*-infested trees (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Miles et al. 1981; Gaunt and Miles 2000; Durán et al. 2016; Monte et al. 2014). ***Eratyrus cuspidatus*** [Tc/L/H] occurs across Mesoamerica, west of the Andes down to northwestern Peru, and along the Venezuelan coastal ranges (Monteiro et al. 2018). Apart from a few records in palms and a reported association with armadillos, there seems to be no information on the wild habitats and hosts of this species; it has occasionally been found in goat corrals or timber piles around houses and once in association with bats in a deserted forest-observation tower (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Abad-Franch et al. 2015). Given the close kinship and clear morphological similarities with *E. mucronatus*, we believe that *E. cuspidatus* may also occupy (mainly moist-forest) large hollow trees. The few *T. cruzi* stocks from both *Eratyrus* species typed to date were all TcI (Brenière et al. 2016; Guhl and Ramírez 2013).

Most Bolboderini are primarily associated with arboreal habitats in moist-forest ecoregions. Their dorsoventrally flattened body may be an adaptation to life under tree bark, between folded dead leaves, or at the base of bromeliads (Monteiro et al. 2018; Carcavallo et al. 1998; Miles et al. 1981; Gaunt and Miles 2000). While *Microtriatoma* and *Parabelminus* (especially *Parabelminus carioca*) may be tentatively seen as putative nest specialists (Sect. 2.3.2), *Belminus*' behavior appears to be closer to that of active-foraging "stalkers." ***Belminus costaricensis*** [–] is known from moist forests in the Sierra de los Tuxtlas, Mexico, where it was collected in a bromeliad epiphyte, and Costa Rica, where the bugs associated with stingless bees and with termites; the holotype was caught on a three-toed sloth (Carcavallo et al.

1985, 1998; Barrett 1991; Dujardin et al. 2002; Sandoval-Ruiz et al. 2012). ***Belminus herreri*** [Tc/H] and ***Belminus laportei*** [−] occur under the loose bark of moist-forest trees in, respectively, Mesoamerica and eastern Amazonia; these morphologically similar bugs may feed on geckos, on arthropods, and likely on small mammals (Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Miles et al. 1981; Sandoval et al. 2004). ***Belminus peruvianus*** [−/H] from the middle-upper Marañón valley of northwestern Peru is the only dry forest-adapted species in its genus; it was found under the bark of a large, hollow *Schinus* tree co-occupied by *R. ecuadoriensis*, a *Didelphis*, and some roosting chickens. In the laboratory, however, newly hatched nymphs refused to bite birds or mammals but fed on geckos and, even more readily, on other triatomines; this may explain why *B. peruvianus* often associates with *Panstrongylus* or *Rhodnius* in houses and trees (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Herrer et al. 1954, 1972; Sandoval et al. 2010). It seems that the only wild ***Belminus rugulosus*** [−/L] on record are two adults found in an *Oenocarpus* palm of the western Magdalena valley montane forests, Colombia (Lent and Wygodzinsky 1979; Carcavallo et al. 1985; Sandoval et al. 2010). ***Belminus corredori*** [−/H] of the eastern Magdalena valley montane forests is unusual for the genus in that adult bugs are light-colored and short-winged; as far as we are aware, this species is only known from a house (Galvão and Angulo 2006; Sandoval et al. 2007). Similarly, ***Belminus ferroae*** [−/H] is only known from houses, this time in the Colombian Cordillera Oriental montane forests; these bugs were mainly feeding on Blattodea cockroaches and only occasionally on rodents, dogs, or humans (Sandoval et al. 2007, 2010). We did not find any data on the ecology of ***Belminus pittieri*** [−] from the moist montane forests on the Venezuelan coastal range; it has the distinctive morphology of the genus, suggesting association with trees and tree bark, epiphytic bromeliads, or other arboreal microhabitats (Lent and Wygodzinsky 1979; Sandoval et al. 2007).

Alberprosenia goyovargasi [−] was found in a decaying tree stump in the dry forest of coastal Venezuela, both under bark and in beetle galleries; it was associated with lizards and snakes but readily feeds on birds and mammals in the laboratory (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002). ***Alberprosenia malheiroi*** [−] has been found twice inside hollow palm stems in southeastern Amazonian rainforests – once with bats in *Oenocarpus* and once in a woodpecker nest in *Euterpe*. In the laboratory, *A. malheiroi* readily feeds on bats, pigeons, and humans, but not on rodents (Abad-Franch et al. 2015; Carcavallo et al. 1995). These limited data are suggestive of a putative "stalker" behavior for the arboreal *Alberprosenia*, but this classification is just tentative.

We regard the two known species of *Cavernicola* as bat-roost specialists; as such, they often occur in bat-inhabited hollow trees (see Sect. 2.4). Temperate-forest populations of *Triatoma lecticularia* and *Triatoma sanguisuga* may associate with squirrels, raccoons, or opossums in hollow trees of the eastern United States (Sect. 2.2.1). Wild *Triatoma huehuetenanguensis* feed on tree-dwelling mammals and birds (Sect. 3.1.2). Adult *Triatoma dimidiata* II invade houses more frequently in communities near forest patches, and there is also evidence that *T. dimidiata* I may

feed on arboreal hosts; this suggests that some populations of this highly versatile "stalker" species may exploit tree habitats in forested ecoregions (Sect. 3.1.2). Some *Panstrongylus* species also use tree microhabitats but seem to be almost invariably associated with vertebrate nests (Sect. 2.3.2.). Finally, several *Rhodnius* species occupy tree hollows or dead logs on occasion (Sects. 2.3.2 and 3.2.2).

3.2.2 Palms

Palms provide primary breeding and foraging habitat for most known *Rhodnius* species (Lent and Wygodzinsky 1979; Carcavallo et al. 1985; Carcavallo et al. 1998; Barrett 1991; Abad-Franch et al. 2015). In particular, palms with large, structurally complex crowns (e.g., in *Attalea*, *Acrocomia*, or *Oenocarpus*) are often infested, although some *Rhodnius* populations exploit smaller-crowned palms such as *Copernicia* in dry ecoregions (Abad-Franch et al. 2015). In line with the view of palm-dwelling *Rhodnius* as opportunistic "stalkers," tight associations between bug and palm species are extremely rare, and many *Rhodnius* species also exploit non-palm microhabitats on occasion (Tables 1 and 2). In this *Section* we follow Henderson et al. (1995) for palm taxonomy.

The northernmost representative of its genus is ***Rhodnius pallescens*** **genotype I** [Tc/L/H] (sensu Abad-Franch et al. 2009; wrongly labeled as *R. pallescens* II in Fig. 2 of Monteiro et al. 2018), which extends from the moist forests of northwestern Colombia into Mesoamerica up to Nicaragua; ***Rhodnius pallescens*** **II** [Tc/L/H] (Abad-Franch et al. 2009) occurs over the middle-lower Magdalena-Cauca Basin and around the Sierra Nevada de Santa Marta in northern Colombia. Wild *R. pallescens* (I/II) are very common in *Attalea butyracea* but have also been found in other palms (*Acrocomia aculeata*, *Elaeis oleifera*) and in a variety of arboreal (tree holes and tree-hole nests; opossum, sloth, and tamandua shelters; squirrel nests) and terrestrial microhabitats (including reports of association with agoutis and armadillos). Blood meal analyses suggest preferential feeding on mammals (mainly opossums, sloths, and tamanduas but also rodents, bats, monkeys, kinkajous, weasels, or raccoons), but birds, lizards, or salamanders may also be fed upon. All *R. pallescens*-derived *T. cruzi* stocks characterized thus far were TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2009, 2015; Gómez-Palacio et al. 2012; Jaramillo et al. 2000; Vásquez et al. 2004; Rodríguez et al. 2018; Kieran et al. 2017; Saldaña et al. 2018; Gottdenker et al. 2011, 2012). The closely related *Rhodnius colombiensis* [Tc/L/H] is endemic to the dry forests of the middle-upper Magdalena valley, where it has only been reported from *A. butyracea*. *Rhodnius colombiensis* is easier to collect from palms occupied by *Didelphis* and has been shown to carry *T. cruzi* TcI (Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2015; Guhl and Ramírez 2013).

Rhodnius ecuadoriensis **I** [Tc/L/H] (sensu Abad-Franch et al. 2009) is endemic to western Ecuador including the moist montane forests on the slopes of the Andes,

the moist lowland and dry coastal forests, and the seasonally dry inter-Andean valleys of southwestern Ecuador and adjacent northwestern Peru. Northern populations live primarily in the also endemic *tagua* ivory palms, *Phytelephas aequatorialis*; in the Andean montane forests, the bugs seem to remain tightly associated with these palms, whereas in the drier lowlands they have also been found in bird (*Campylorhynchus*), squirrel (*Simosciurus*), opossum (*Didelphis*), and rodent (*Mus*) nests built on trees (see Sect. 2.3). There is one record from the African oil palm, *Elaeis guineensis*, in association with *Didelphis* in lowland moist forest. Coastal *R. ecuadoriensis* I often carry *T. cruzi* TcI (Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Abad-Franch et al. 2001, 2015, 2009; Suárez-Dávalos et al. 2010; Costales et al. 2015). Southern *R. ecuadoriensis* I (from the Andean valleys along the western Ecuador-Peru border), as well as *R. ecuadoriensis* II (from the central-northern Peruvian Andes), seem to have completed the transition from palms to arboreal-nest microhabitats – and both seem to largely behave as nest specialists (Sect. 2.3.2).

Rhodnius pictipes [Tc/L/H] is widespread across the Orinoco-Amazonas system, where it occurs in at least ten palm species and also, occasionally, in epiphytic bromeliads and the nests of birds or *Didelphis*. In palms, bugs often associate with opossums (*Didelphis*, *Marmosa*, *Caluromys*) but may also feed on anteaters, porcupines, bats, birds, or squamate reptiles. *Rhodnius pictipes* can be fairly bold; they often carry *T. cruzi* TcI, but a few stocks from eastern Amazonia were typed as TcII and TcIII/TcIV (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2015; Miles et al. 1981, 1983). The ecology of an undescribed species superficially similar to, yet genetically distinct from, *R. pictipes* found in the Sierra Nevada de Santa Marta, northern Colombia (but perhaps also present in northwestern Venezuela), remains unknown (Monteiro et al. 2018; Abad-Franch et al. 2009; Aldana et al. 2003).

Rhodnius stali [Tc/L/H] from the southwestern Amazon moist forests and transitional ecoregions (montane Yungas, Chiquitano dry forests, and Beni-Cerrado-Pantanal savannas) is also close to *Rhodnius pictipes*. It is common in *Attalea phalerata* palms but may also occur in *Oenocarpus*, *Astrocaryum*, and *Copernicia* and has been shown to infest the arboreal, woven-stick nests of coatis (*Nasua*) in the Pantanal flooded savanna (Tables 1 and 2). Wild *R. stali* may feed on birds, coatis, rodents, or opossums and have been found infected with *T. cruzi* TcI (Monteiro et al. 2018; Carcavallo et al. 1998; Georgieva et al. 2017; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2015; Abad-Franch et al. 2009; Santos et al. 2015, 2019; Justi et al. 2010). ***Rhodnius amazonicus*** [–/L/H], another member of the *Rhodnius pictipes* lineage (Monteiro et al. 2018), is only known from a few adult specimens collected at light and invading houses in the central-eastern Amazon; the natural habitats and hosts of the species remain unknown, but it is, in all likelihood, primarily associated with palms (Monteiro et al. 2018; Abad-Franch et al. 2009; de Almeida et al. 1973; Bérenger and Pluot-Sigwalt 2002; Castro et al. 2010; Rosa et al. 2017).

Unusually for a member of the *Rhodnius pictipes* lineage (and for the genus), the morphologically distinct ***Rhodnius brethesi*** [Tc/L] appears to be tightly associated with a single palm species – the *piaçava*-fiber palm, *Leopoldinia piassaba*, which is endemic to the sandy-soil Rio Negro Campinaranas of northern Amazonia. The bugs live among the fibers that cover the palms' stems, where they seem to feed mainly on geckos (*Techadactylus* and perhaps *Gonatodes*) and rodents. *Rhodnius brethesi* often attack forest workers in *piaçava* stands; an adult bug was once recorded attempting to feed on a moth on the white cloth of a light trap. *Trypanosoma cruzi* TcI, TcIV, and, more rarely, TcIII have been found infecting *R. brethesi* (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Abad-Franch et al. 2009; Mascarenhas 1991; Monte 2010).

The dry-to-semiarid ecoregions around the Maracaibo Basin and the northern end of the Colombian/Venezuelan Andes provide habitat to the little-known ***Rhodnius neivai*** [Tc/H]. In the wild, this species has been collected in palms (*Copernicia* and *Attalea*) and inside dead, hollow trees (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Abad-Franch et al. 2015; Véliz et al. 1972).

Rhodnius prolixus [Tc/L/H] is one of the most important domestic vectors of *T. cruzi*. Wild populations occur across the Orinoco Llanos and in adjacent ecoregions east of the Andes – the semiarid Venezuelan coast and the dry montane forests on the eastern slopes of the northern Andes (Monteiro et al. 2018; Abad-Franch et al. 2009). Wild *R. prolixus* populations occur in the crowns of at least ten palm species but have also been found on trees and in bromeliad epiphytes (Tables 1 and 2). In the Llanos, the bugs often infest the small-crowned *Copernicia tectorum* to the north-northeast and the large-crowned *Attalea butyracea* to the south-southwest. Wild *R. prolixus* have been reported in association with birds including wrens (*Thryothorus*, *Troglodytes*), warblers (*Setophaga*), ovenbirds (*Phacellodomus*), mockingbirds (*Mimus*), flycatchers (*Cnemotriccus*), storks (*Ciconia*, *Jabiru*, *Mycteria*), herons (*Agamia*), ibises (*Cercibis*, *Theristicus*), chachalacas (*Ortalis*), geese (*Neochen*), caracaras (*Caracara*, *Milvago*), kites (*Elanoides*), buzzards (*Buteo*), and black hawks (*Buteogallus*); with mammals including opossums (*Didelphis*, *Marmosa*, *Philander*), anteaters (*Tamandua*), skunks (*Conepatus*), raccoons (*Procyon*), rodents (*Coendou*, *Proechimys*, *Oecomys*, *Oligoryzomys*, *Zygodontomys*, *Dasyprocta*, *Cuniculus*), bats (*Artibeus*, *Myotis*), and armadillos (*Dasypus*); and with lizards (*Ameiva*, *Iguana*) and geckos (*Hemidactylus*). All *T. cruzi* stocks from wild *R. prolixus* characterized so far were TcI, except for one TcIV from an allegedly sylvatic Colombian bug (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2009; Di Iorio and Turienzo 2009; Monteiro et al. 2003; Gamboa 1961; Feliciangeli and Torrealba 1977; Rendón et al. 2015; Urbano et al. 2015; Barnabé et al. 2000; Erazo et al. 2019).

Rhodnius robustus **genotype I** [Tc/L/H] (sensu Monteiro et al. 2003, 2018) is sister to *R. prolixus* and appears to be endemic to the moist montane forests of the

Cordillera de Mérida in northwestern Venezuela, although it extends into the adjacent Maracaibo (west) and Apure-Villavicencio (east) dry forests. Wild *R. robustus* I occur in palms (*Attalea butyracea*, *Acrocomia aculeata*, and possibly *Oenocarpus bataua*) and are often infected with *T. cruzi* (likely TcI and TcIV); one report suggests association with *Didelphis* in *Acrocomia* (Abad-Franch et al. 2009; Monteiro et al. 2003, 2018; Lent and Valderrama 1973; Galíndez-Girón et al. 1994; Feliciangeli et al. 2002; Longa and Scorza 2005; Fitzpatrick et al. 2008; Pavan and Monteiro 2017; Pavan et al. 2013). ***Rhodnius robustus* V** [−] is known only from a few specimens collected in *Attalea speciosa* palms of the central Amazon moist forests on the south bank of the lower Negro River (Monteiro et al. 2018; Abad-Franch et al. 2009; Abad-Franch and Monteiro 2007).

Rhodnius robustus* IV** [Tc/L/H] (Monteiro et al. 2003) occurs across the Guiana shield moist forests and extends into northern Venezuela and the northern-central Amazon; it has also been found just south of the Amazon River mouth. Bugs identified as *R. robustus* from these subregions carry *T. cruzi* TcI and TcIV and have been recorded in *Attalea maripa*, *Acrocomia aculeata*, *Astrocaryum murumuru*, *As. aculeatum*, *Mauritia flexuosa*, and *M. carana*, as well as in epiphytic bromeliads. One record suggests possible association with *Caluromys philander* in *A. maripa* (Monteiro et al. 2018; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2009; Miles et al. 1981, 1983; Lent and Valderrama 1973; Pavan and Monteiro 2017; Pavan et al. 2013; Abad-Franch and Monteiro 2007; Ricardo-Silva et al. 2012). ***Rhodnius marabaensis [Tc/L/H] is probably what Monteiro et al. (2003) called *R. robustus* III (Monteiro et al. 2018). It is closely related to *R. robustus* IV and occurs in moist forests of southeastern-central Amazonia (south of the Amazonas-Solimões main channel), as well as in the transition to adjacent dry forest and savanna; the bugs occur in *Attalea speciosa* and probably also in other palms including *A. maripa* or *Acrocomia aculeata* (Monteiro et al. 2003, 2018; Pavan and Monteiro 2017; Pavan et al. 2013; Gurgel-Gonçalves et al. 2008). ***Rhodnius montenegrensis*** [Tc/H] (formerly *R. robustus* II sensu Monteiro et al. 2003; see also Monteiro et al. 2018; Brito et al. 2019) is widespread across western and southern Amazonia and has been found in *Attalea butyracea*, *A. speciosa*, *A. maripa*, and *A. phalerata* (with a *Tamandua* anteater); it may also occur in *Astrocaryum*. Bugs genotyped as *R. robustus* II or collected within *R. montenegrensis*' range carry *T. cruzi* TcI, TcIV, and perhaps TcV (Monteiro et al. 2018; Carcavallo et al. 1985; Carcavallo et al. 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2009; Pavan and Monteiro 2017; Pavan et al. 2013; Dias et al. 2010; Dias et al. 2014; Bilheiro et al. 2019).

Rhodnius barretti [Tc/L/H] is known only from the Napo moist forests of western Amazonia (Monteiro et al. 2018), where it has been collected in *Attalea butyracea* and *Oenocarpus bataua* but may also occur in other large palms (*Astrocaryum*, *Elaeis*). Hungry *R. barretti* will readily stalk humans working on palm crowns during the day; the bugs were also seen attempting to bite the sun-heated upper end of a fiberglass ladder used to climb the palms (Abad-Franch et al. 2013 and FA-F, pers.

obs.). *Rhodnius barretti* has been reported to feed on *Cebus* capuchins and *Saimiri* squirrel monkeys and is often infected with *T. cruzi* (Georgieva et al. 2017; Abad-Franch et al. 2013). ***Rhodnius dalessandroi*** [–/H] was collected only once from *Oenocarpus bataua* palms and nearby houses in the transition between the southwestern end of the Orinoco Llanos and the moister forests of western Amazonia (D'Alessandro et al. 1971).

The seasonally dry Cerrado savannas of central Brazil are home to ***Rhodnius neglectus*** [Tc/L/H], which may be primarily associated with *Acrocomia aculeata* and *Syagrus oleracea* but also infests several other palm species including *Attalea* (also as *Rhodnius milesi*), *Oenocarpus*, or *Copernicia*. *Rhodnius neglectus* occurs in bird (*Anumbius* and *Phacellodomus* ovenbirds, *Mimus* mockingbirds, *Gnorimopsar* blackbirds, *Caracara* caracaras) and *Didelphis* nests in *Mauritia flexuosa* palm crowns, which in the absence of nests are rarely infested by this species. Wild *R. neglectus* may occur in clumps of agaves and on trees and (apart from birds) may feed on opossums, rodents, and bats. The bugs have been found infected with *T. cruzi* TcI and TcII (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Brenière et al. 2016; Izeta-Alberdi et al. 2016; Abad-Franch et al. 2009; Gurgel-Gonçalves et al. 2012a; Gurgel-Gonçalves et al. 2004, 2008; Valente et al. 2001).

Rhodnius nasutus [Tc/L/H] is endemic to the semiarid Caatinga and the moister Atlantic Forest Caatinga enclaves. The bugs associate with *Copernicia tectorum* in the former and with *Attalea speciosa* in the latter but may also occur in *Acrocomia*, *Syagrus*, and *Mauritia*, as well as, occasionally, on *Licania* trees; they can, in addition, infest bird nests (ovenbirds, *Pitangus* kiskadees) and feed on opossums, rodents, squamate reptiles, or amphibians. *Rhodnius nasutus* has been found infected with *T. cruzi* TcI (Lent and Wygodzinsky 1979; Monteiro et al. 2018; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Georgieva et al. 2017; Rabinovich et al. 2011; Abad-Franch et al. 2009; Lima and Sarquis 2008; Dias et al. 2008, 2011; Cura et al. 2010). One population genetically close to *R. nasutus* occurs in *Attalea maripa* palms of the transition between the Guiana savanna and piedmont moist forests in Roraima, Brazil – that is, north of the Amazon Basin (Monteiro et al. 2018).

Rhodnius domesticus of the moist Brazilian Atlantic forests is the southernmost representative of its genus; it has been reported from *Attalea* palms, yet most records refer to mammal nests built in hollow trees or among bromeliads – either terrestrial or epiphytic (see Sect. 2.3.2). Sampling of 110 palms within *R. domesticus*' range in eastern Bahia yielded no bugs of this species (Gurgel-Gonçalves et al. 2012b). We note that *Rhodnius zeledoni* is in all likelihood a synonym of *R. domesticus* (Monteiro et al. 2018).

3.2.3 Epiphytes

Triatoma tibiamaculata [Tc/L/H] is one of the few triatomine-bug species that primarily associate with epiphytes; it is endemic to the Brazilian moist Atlantic Forest and has been described as "exquisitely adapted" to *Aechmea* bromeliad epiphytes, with a "pinkish camouflage that ideally suits its habitat at the junction between the epiphyte and the host tree" (Gaunt and Miles 2000, p. 557 and 559). Opossums (*Didelphis*, *Marmosa*) and rodents (spiny and rice rats) use the same bromeliad habitats. There are, in addition, a few records of *T. tibiamaculata* in *Attalea* palms. Bugs caught invading houses near urban forest fragments were reported to have fed mainly on birds, opossums, and rodents and were very often infected with *T. cruzi* TcII and TcI (Lent and Wygodzinsky 1979; Miles 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002; Gurgel-Gonçalves et al. 2012b; Ribeiro Jr et al. 2015). Other triatomines that make use of epiphytic microhabitats are the *Microtriatoma* plus some species of *Belminus*, *Parabelminus*, *Triatoma*, *Panstrongylus*, and *Rhodnius* (Table 1). Although the evidence is sparse, we believe that ***Parabelminus yurupucu*** [–] might be regarded as another active-foraging "stalker" that primarily exploits epiphytic-bromeliad microhabitats (*Aechmea*, *Hohembergia*) in association with small vertebrates including rodents, frogs, or lizards – on which the bugs were reportedly feeding (Lent and Wygodzinsky 1979; Carcavallo et al. 1985, 1998; Barrett 1991; Dujardin et al. 2002).

4 Closing Remarks

The Triatominae have adapted to extremely diverse ecoregions, from hyperarid deserts to hyper-moist rainforests, throughout the southern Nearctic and Neotropical realms (Monteiro et al. 2018). There, the bugs occupy similarly diverse terrestrial and arboreal habitats/microhabitats (Gaunt and Miles 2000; Zeledón and Rabinovich 1981). Remarkably, almost exactly half (51.8%) of the 143 species or populations we evaluated (those in Table 1's "primary association" column, but excluding *Triatoma rubrofasciata* and two undescribed *Rhodnius* species) are primarily terrestrial, and 46.9% are primarily arboreal – with the bat roost-specialized *Cavernicola* accounting for the remaining ~1.3%. Most terrestrial taxa belong in the Triatomini, whereas the Rhodniini, the Alberprosenini, and the Bolboderini (with the possible exception of *Bolbodera scabrosa*) all exploit tree-related microhabitats (Fig. 1). Within the Triatomini, the earliest-diverging lineage is composed of five host-generalist "stalker" species – the primarily arboreal *Triatoma dispar*, *T. venosa*, *T. nigromaculata*, and *T. carrioni*, plus the terrestrial *T. boliviana* (Fig. 2). Nearly 75% of the remaining Triatomini (72% in the "North American" lineage and 73% in the South American lineage sensu Monteiro et al. 2018) are primarily terrestrial; most of the taxa that reversed to arboreal habitats in this group belong to the genus *Panstrongylus* (in which three species adapted back to terrestrial habitats), and the rest (two *Eratyrus* and six *Triatoma* species) are scattered across the phylogeny

(Fig. 2). Andean, stony ground-dwelling *Triatoma infestans* probably evolved from a relatively recent arboreal ancestor (Fig. 2) that lived in the dry lowlands corresponding to the contemporary Gran Chaco.

Our evaluation also suggests that most American triatomine-bug taxa (71.3%) are putative host-generalist, opportunistic "stalkers," vs. just 27.3% "sit-and-wait," nest-specialized species; again remarkably, this ~70/30% share is consistent across the most speciose tribes – the Triatomini, the Rhodniini, and the Bolboderini (Fig. 1). Nest specialists are particularly rare among the truly South American Triatomini (sensu Monteiro et al. 2018), with just two closely related species, *Triatoma platensis* and *T. delpontei*, specialized in arboreal bird nests (Figs. 1 and 2). Within the "North American" lineage (sensu Monteiro et al. 2018), nest specialists outnumber opportunistic "stalkers" in the truly North American clade (where most taxa specialize in woodrat nests in dry-to-arid environments) and among the *Panstrongylus* – in which a single taxon, *Triatoma tibiamaculata* (see Monteiro et al. 2018), fairly clearly behaves as a putative "stalker." Particularly in semiarid-to-arid ecoregions, the northernmost representatives of the *Triatoma phyllosoma* species group (*Triatoma sanguisuga* and *T. indictiva*) seem to have "copied" the strategy used by most North American triatomines in the same areas – nesting woodrat parasitism (Figs. 1 and 2).

Taken together, these observations point toward an arboreal, "stalker" lifestyle for the ancestor of the Triatominae, with nest specialization (the "sit-and-wait" strategy as defined here) independently arising a few times in each of the major lineages (Fig. 2). In particular, our appraisal suggests that triatomines may derive from a "stalker," opportunistic reduviid that preferentially hunted within arboreal vertebrate nests – much in the way *Microtriatoma trinidadensis* does today (Hwang and Weirauch 2012; Barrett 1991). Most extant reduviids, in fact, are generalist active hunters that move about their habitats to stalk invertebrate prey, and a few associate with vertebrate nests (Schuh and Slater 1995). In the Triatomini, adaptation to terrestrial habitats likely happened after the divergence of the *Triatoma dispar* lineage, and over 70 descendant taxa retain this trait across the entire geographical range of the Triatominae in the Americas (Figs. 1 and 2). This adaptive novelty, therefore, may have played a key role in the diversification of the Triatomini across Nearctic and Neotropical dry, open ecoregions; some sub-lineages, however, shifted back to arboreal habitats – mammal tree nests (*Panstrongylus*) or trees themselves (*Eratyrus*, six terminal *Triatoma* taxa, and the ancestor of the three-species *Triatoma infestans* group) (Fig. 2).

The "sit-and-wait" nest-specialist lifestyle has evolved in both arboreal (22% of taxa) and terrestrial triatomines (34%); overall, most nest-specialized taxa are arboreal (59%) and most "stalkers" are terrestrial (59%) (Fig. 2). Parallel shifts to nest specialization apparently involved a series of parallel phenotypic adaptations in different triatomine-bug lineages (Box 1). For example, "sit-and-wait" nest specialists tend to be smaller than their "stalker" counterparts, perhaps with the exception of *Panstrongylus* species associated with the nests of relatively large vertebrates such as armadillos or opossums (Fig. 3). The heads of nest-specialist bugs are also particularly short and stout; genetic data suggest (cf. Monteiro et al. 2018) that the

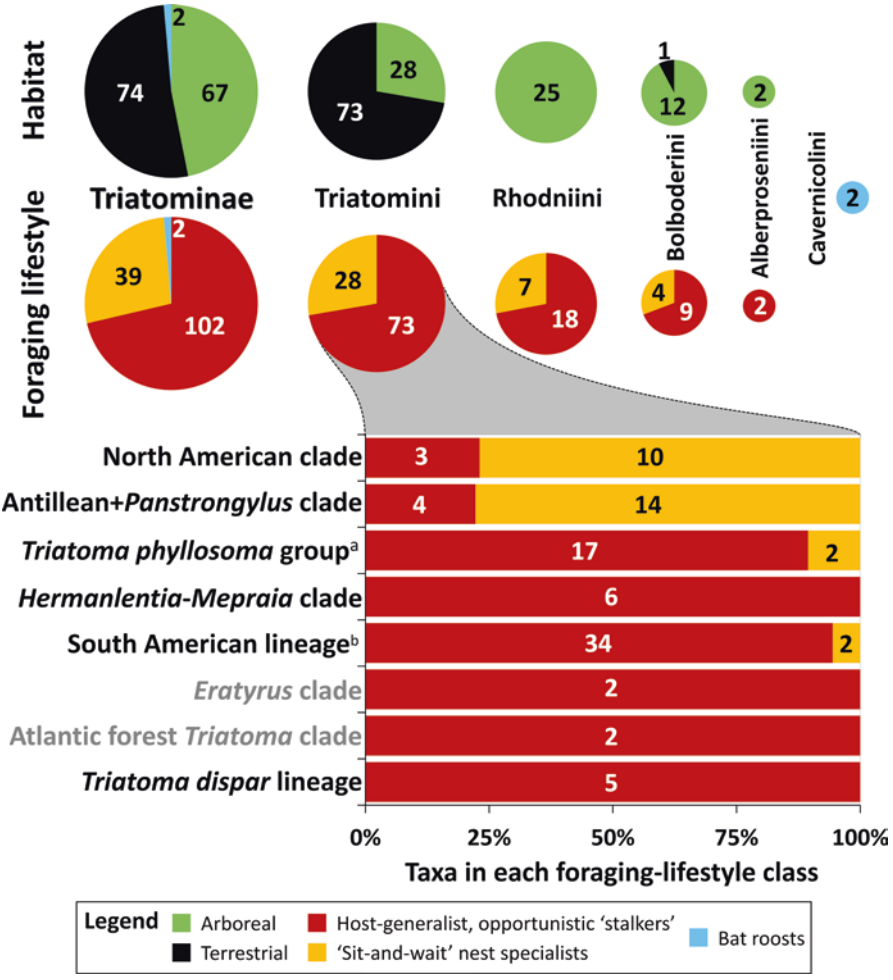

Fig. 1 The habitats and foraging lifestyles of wild Triatominae in the Americas: distribution across major systematic subdivisions. Graph color codes are given in the inset legend. Areas in graphs represent the proportions of taxa in each habitat or behavior class; actual taxon numbers are also presented. [a] Excluding the Old-World Triatominae, which were omitted from the present assessment. [b] Excluding *Eratyrus* and the Atlantic Forest *Triatoma* (in gray font right below). The systematic arrangement follows Monteiro et al. (2018)

long-headed *Triatoma tibiamaculata* is an atypical *Panstrongylus* that switched back to a "stalker" strategy in canopy epiphytes of the Brazilian Atlantic Forest (Fig. 3). A counterexample may be the extremely small-bodied, short-headed *Alberprosenia*, which we tentatively classify with the "stalkers" in spite of the limited evidence. Host-generalist "stalkers," in addition, tend to be bolder, as well as swifter movers (both at approaching hosts and at escaping or hiding from them), than "sit-and-wait" nest specialists. More generally, one may expect that the sensory

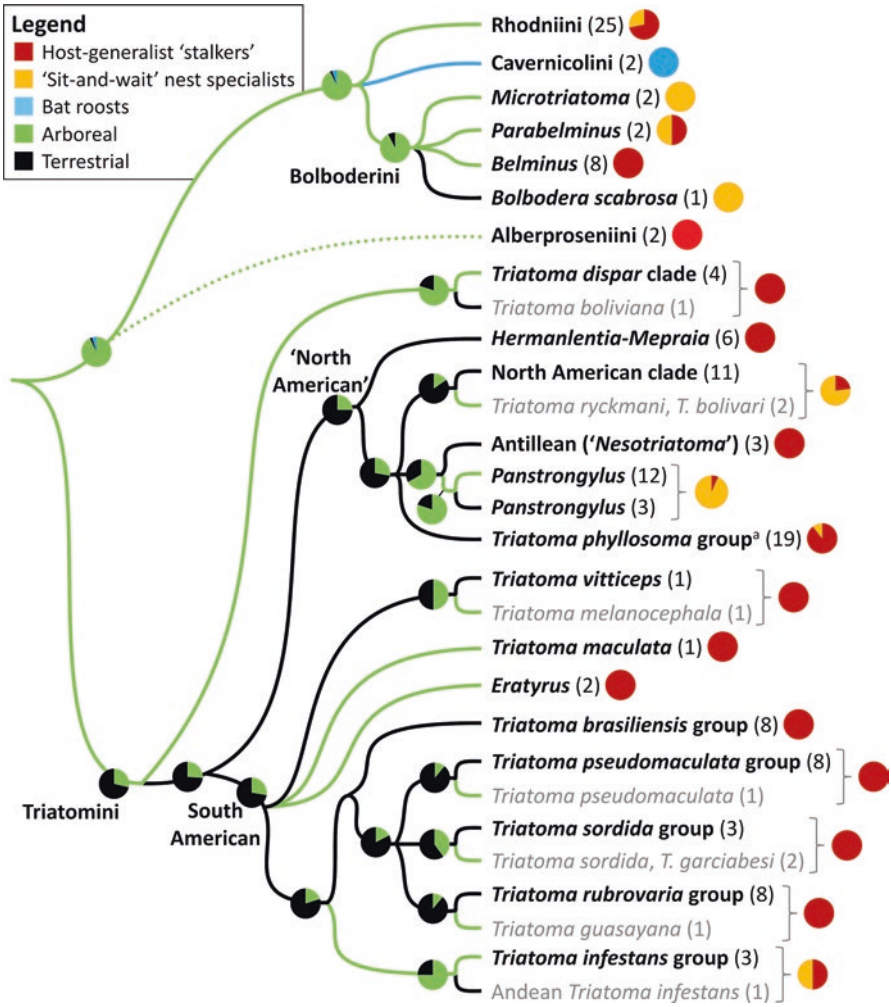

Fig. 2 The habitats and foraging lifestyles of wild Triatominae in the Americas: distribution on a (schematic) phylogeny. Branch and pie graph color codes are given in the inset legend. The number of taxa in each terminal branch is given in parentheses. Major systematic subdivisions are indicated across the rough, schematic phylogeny (based on Monteiro et al. 2018). Uncertainties regarding the phylogenetic position and foraging lifestyle of the Alberproseniini are highlighted, respectively, by a dotted branch and a lighter-red pie graph. Individual species diverging in habitat from the majority of species in their terminal clade or group are in gray font. [a] Excluding the Old-World Triatominae, which were omitted from the present assessment

system of "stalker" species is adapted to relatively long-range host detection, and their nervous and locomotor systems adapted to "host-chasing." "Sit-and-wait" specialists, on the other hand, may be expected to have a more highly specialized saliva chemistry than opportunistic "stalkers" – which are, in general, more catholic feeders. This might help explain why the bites of nest-specialist triatomines are often

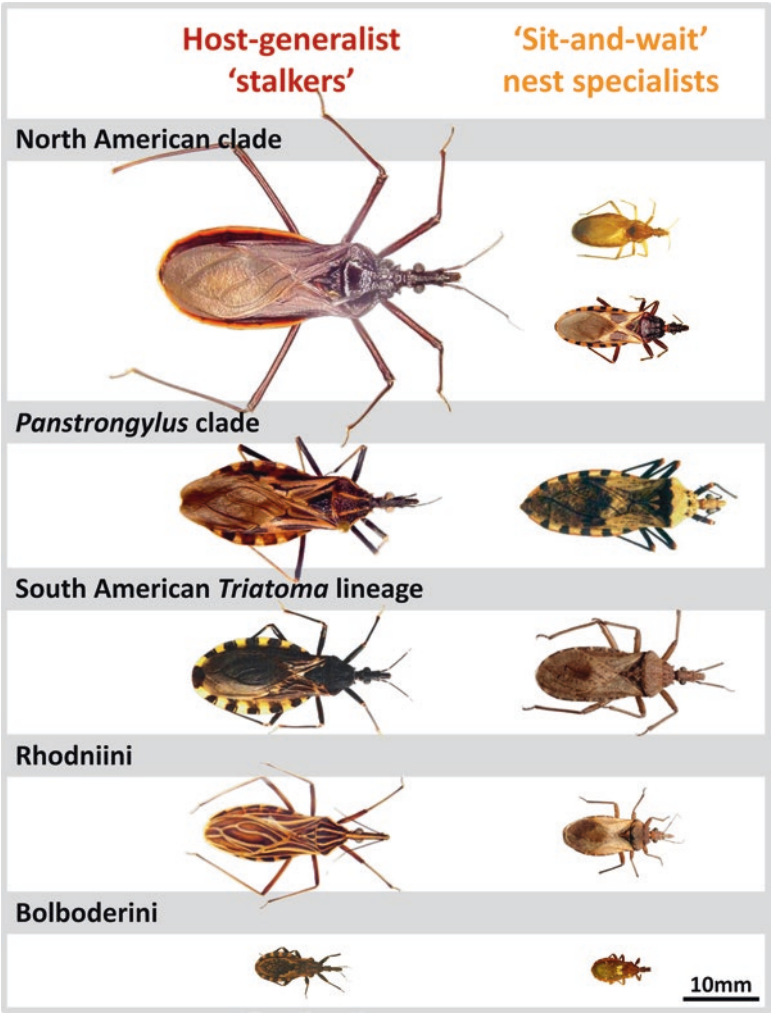

Fig. 3 The foraging lifestyles of wild Triatominae in the Americas: an illustration of phenotype trends. From left to right and from top to bottom: *Dipetalogaster maxima*, *Paratriatoma hirsuta* and *Triatoma neotomae*; *Triatoma tibiamaculata* and *Panstrongylus geniculatus*; *Triatoma infestans* and *Triatoma platensis*; *Rhodnius robustus* (*s.l.*) and *Psammolestes coreodes*; and *Belminus ferroae* and *Microtriatoma trinidadensis*. Note that all bugs are adults and are on the same (approximate) size-scale (see scale bar on the bottom-right corner). Clades and lineages follow Monteiro et al. (2018)

painful and can trigger strong allergic reactions – to the point that several woodrat nest-adapted North American triatomines are of public health concern because their bites can produce anaphylaxis (Bern et al. 2011, 2020) (Box 1).

We detected no pattern of association between *Trypanosoma cruzi* discrete typing units (DTUs) found circulating in wild cycles and the vectors' primary habits or

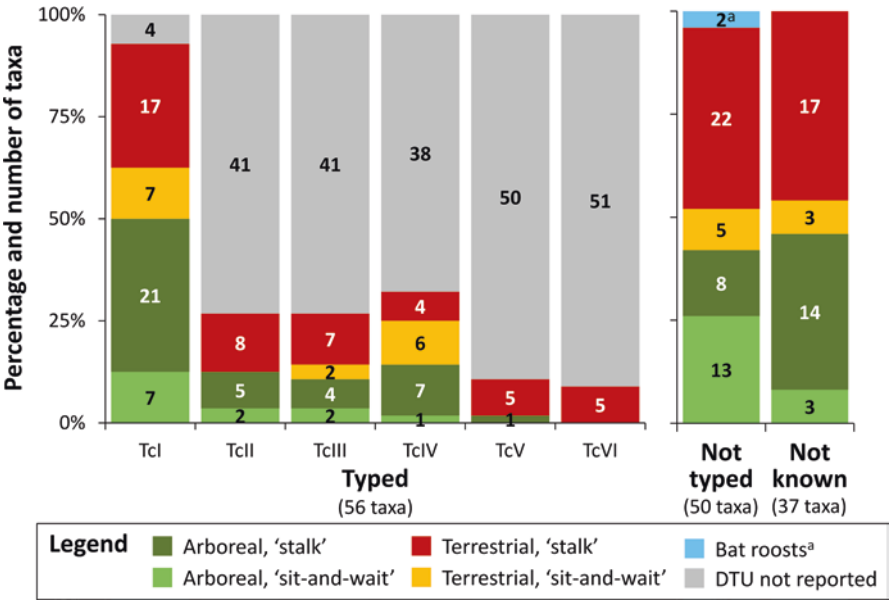

Fig. 4 *Trypanosoma cruzi* discrete typing units (DTUs) circulating among wild triatomine bugs in the Americas. The left hand-side panel shows the absolute numbers and percentages of triatomine-bug taxa found carrying each DTU (TcI to TcVI) in the wild (in each bar, $n = 56$ taxa for which we found DTU data). The right hand-side panel separately shows data for bug taxa in which *T. cruzi* parasites were detected but not typed ($n = 50$ taxa), or were not found or not reported ($n = 37$ taxa). [a] *Cavernicola pilosa* carries *T. cruzi* TcBat and *T. cruzi marinkellei*, which is probably also transmitted by *C. lenti*

habitats (Fig. 4). Instead, our appraisal shows that the evidence is clearly too sparse to draw meaningful conclusions (Brenière et al. 2016; Izeta-Alberdi et al. 2016). For example, of the 52 triatomine-bug taxa in which the most widespread DTU (TcI) has been found, 53.9% are arboreal and 46.2% are terrestrial; this is hardly suggestive of any TcI/habitat association (Gaunt and Miles 2000). For the rest of DTUs, which are much rarer, the data so far available are even less informative (Fig. 4).

From the practical standpoint of Chagas disease epidemiology, we finally note that the most important domestic vectors of *T. cruzi* – *Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiata* – are typical host-generalist, opportunistic "stalkers" (Box 1). The fourth historically major vector, *Panstrongylus megistus*, used to quite heavily infest extremely poor, mud/stick-walled, thatch-roofed huts such as those described by Carlos Chagas 110 years ago (Chagas 1909); however, more recent reports of house infestation by *Panstrongylus* species including *P. megistus* often (but not always) refer to relatively small colonies (Patterson et al. 2009; Carrasco et al. 2014; Martins et al. 2006; Forattini et al. 1977; Reyes-Lugo and Rodríguez-Acosta 2000; Feliciangeli et al. 2004; Waleckx et al. 2015). More broadly, only a few putative "sit-and-wait" nest specialists – *Triatoma barberi*;

some populations of *Panstrongylus megistus*, *P. rufotuberculatus*, *P. geniculatus*, *P. chinai*, or *P. lignarius*; and southern *Rhodnius ecuadoriensis* – rank among the most medically relevant triatomine-bug taxa (Box 1). This suggests that, although there are important exceptions, "stalker" species might be overall more likely to thrive in man-made habitats than nest-specialist species. For one thing, opportunistic "stalkers" are already adapted to feed upon the potentially diverse vertebrate hosts co-occurring with them in shared microenvironments. Here, we recall, "feeding" has to be understood as a fairly complex process that involves locating and reaching a suitable host, biting it without triggering a (potentially lethal for the bug) defensive response, sucking a share of its blood, and then swiftly retreating to a safe hideout (Lazzari et al. 2013). Host-generalist "stalker" species might then be, in a sense, preadapted to life in man-made habitats in a way in which "sit-and-wait" nest-specialist species are not (Box 1).

We believe, in sum, that our "foraging-lifestyle hypothesis" may shed new light on some little understood, and at times somewhat perplexing, aspects of the bugs' systematic-ecological, morphological, physiological, and behavioral diversity – possibly including variations in life history traits crucial to medical relevance (Box 1). We expect that this review will stimulate, and perhaps guide, future research on the underpinnings of such diversity. Ultimately, we hope that, by introducing a fresh perspective on triatomine-bug ecology and behavior, this work will contribute to the development of improved strategies for the prevention of vector-borne Chagas disease.

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Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma cruzi* in Domestic Habitats

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Abstract Triatomine species largely differ in their degrees of adaptation to thrive in domestic habitats, blood-feed on humans, and transmit *Trypanosoma cruzi*. Pathogen transmission dynamics are shaped by ecological, biological, and social factors. Here we link housing quality and host availability to the host-feeding patterns of domestic triatomines and examine how their blood-feeding performance affects temperature-dependent vital rates and bug population dynamics. The stability/instability habitat divide connects with the large/small triatomine population size dichotomy and on whether bug population dynamics are density-dependent or density-independent and dominated by stochasticity. Seasonal variations in temperature acting on triatomine blood-feeding activity and human-vector contact rates determined the spring peak of symptomatic acute cases of Chagas disease in northern Argentina across four decades. The presence of domestic animals (dogs, cats, and chickens) and commensal rodents increases domestic infestation, vector infection, and parasite transmission across multiple settings and triatomine species. Both ecological and social factors contribute to human infection risk through social vulnerability, mobility patterns, and housing instability. Understanding the interactions among eco-bio-social factors may lead to the design and implementation of improved, sustainable disease control or elimination strategies.

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1 Background

The transmission of zoonotic and vector-borne diseases may be considered an inherently ecological process involving intraspecific and interspecific interactions between vectors, pathogens, and host populations (Keesing et al. 2006). When human populations are implicated, pathogen transmission dynamics also involve socioeconomic, cultural, political, psychological, and ethical factors that pertain to the human dimension of disease (Spiegel et al. 2005; Briceño-León 2009; Ellis and Wilcox 2009). These factors may be classified as intrinsic or extrinsic to the human population (Ehrenberg and Ault 2005). Intrinsic factors are biological in nature (e.g., immune response) and can only be manipulated by advances in medical research and technology. Extrinsic factors include the environmental context, vector ecology and behavior, human activities, socioeconomic inequalities, and political factors, among others. These factors operate and interact at different scales within a complex system characterized by multiple interdependent components featuring feedback loops and nonlinear relations (Meadows 2008).

A thorough understanding of the combined effects of these factors is required to design more effective and sustainable disease control interventions (Charron 2012). Control interventions have traditionally been crafted in a reductionist biomedical approach, which argues that the sum of information provided by separately studying each component of the system is sufficient to understand disease transmission dynamics (El Sayed et al. 2012). Other more integrative approaches (ecohealth or eco-bio-social) focus on the interactions among multiple ecological, biological, and social factors and their combined effects on human health (Spiegel et al. 2005; Charron 2012). Social factors include large-scale forces such as poverty and social inequality; land tenure and agricultural development; public and private services such as water supply, sanitation, and garbage collection; demographic change and urbanization; vector control programs and other healthcare services; and community- and household-based knowledge, attitudes, and practices. The more integrative approaches pursue the design and implementation of sustainable, cost-effective disease control strategies to reduce social- and gender-associated inequalities related to health (Charron 2012; WHO 2008).

Chagas disease, caused by the protozoan *Trypanosoma cruzi* and mainly transmitted by triatomine bugs, is a major neglected tropical disease and a serious cause of human chronic disease in the Americas (WHO 2015). All triatomine species and mammals appear to be susceptible to the infection. Triatomine bugs are obligate hematophagous insects with opportunistic feeding habits on mammals and birds, and while doing so, they may contaminate their skin with urine or feces and transmit *T. cruzi* to mammalian hosts. The parasite may also be transmitted through food

items contaminated by sylvatic triatomine bugs or sylvatic hosts (e.g., opossums). Other transmission routes (vertical, transfusional, organ transplant) exist. Some triatomine species may also transmit other trypanosomatids (e.g., *Trypanosoma rangeli*) to humans and mammals through their bites; *T. rangeli* is considered nonpathogenic for its mammalian hosts.

Here we selectively review the biological, ecological, and social factors involved in the domestic transmission of *T. cruzi* (Fig. 1). We frequently provide examples related to the Southern Cone of South America and to its main vector *Triatoma infestans*, partly because this is where Chagas disease attained the greatest prevalence in humans (WHO 2015) and partly because of the wealth of information on *T. infestans*. We also examine how ecological and social factors contribute to human infection risk. We defer consideration of vector control measures and their impacts to a separate chapter. Disease-related aspects and spatial dynamics fall outside of the scope of this review.

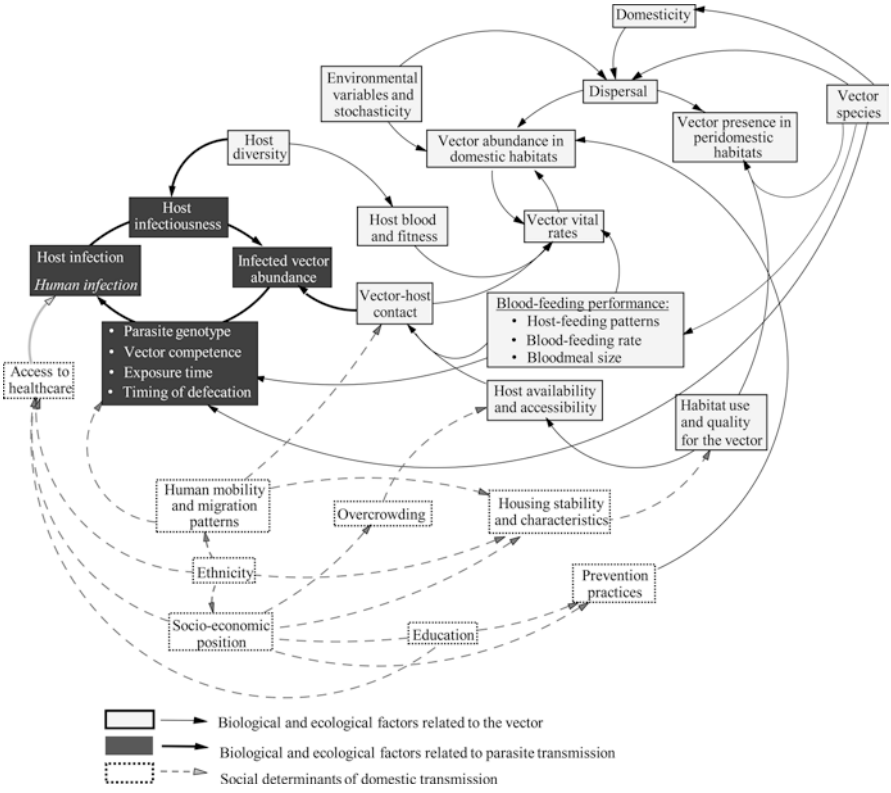

Fig. 1 Conceptual map of the relations between ecological, biological, and social factors affecting the domestic transmission of *T. cruzi*

2 Biological and Ecological Factors Related to the Vector

2.1 Species and Epidemiologic Relevance

Of the approximately 150 species of Triatominae (Heteroptera: Reduviidae) currently recognized, <10 species have become closely adapted to thrive in domestic premises and feed on humans and other domestic animals (i.e., domesticated), and < 20 species have been involved in the transmission of *T. cruzi* infection to humans (Gourbière et al. 2012). Noireau and Dujardin (2010) divided triatomines in domestic, domiciliary, intrusive, and sylvatic species based on their epidemiological relevance for transmission and control. Although several attempts have been made to classify triatomine species, the subject still remains controversial (e.g., Waleckx et al. 2015a; Abad-Franch 2016) (see chapter "Chagas Disease Vector Control" on vector control). In this chapter, "domestic or domiciliary" refers to the set of contiguous human sleeping quarters and rooms that share a continuous roof structure.

2.2 Domesticity and Vector Abundance

Domesticity conveys several selective fitness advantages related to stable habitats (more protected from exposure to climatic extremes, such as human dwellings and structurally similar outhouses) with a stable supply of hosts (humans and domestic or synanthropic animals), balanced by the costs of adaptation to diverse types of habitats, blood-feeding on domestic hosts, and progressive genetic simplification (Dujardin and Schofield 2004). The immediate gains of domesticity translate into much larger triatomine population sizes than in sylvatic habitats, with additional benefits derived from human-mediated passive dispersal and an enlarged geographic range. Nondomestic triatomine species occupying peridomestic and sylvatic ecotopes are well adapted to cope with habitat instability.

Triatoma infestans expresses the extreme of the evolutionary trend toward domesticity (Dujardin and Schofield 2004), with widespread sylvatic foci only in Bolivia and Chile (see chapter "Chagas Disease Vector Control"). This dual feature is also shared by *T. dimidiata* in Central America versus Ecuador and by *Rhodnius prolixus* in Venezuela and Colombia versus Central America and southern Mexico. The intimate adaptation of *T. infestans* and *R. prolixus* to domestic habitats and epidemiological significance as vectors of human *T. cruzi* infection, combined with other technical aspects, justified launching regional elimination programs (WHO 2002).

Illustrating their relative success, the top domestic abundances recorded in a single human habitation reached 7900 (Rabinovich et al. 1979) to 11,403 *R. prolixus* (Sandoval et al. 2000a, b) and 8500 *T. infestans* over a 3-year period (Dias and Zeledón 1955). An enclosed experimental population of *T. infestans* in a hut of ~1 m^3 with one chicken reached 15,844 insects (excluding eggs) 2 years after being

founded with 5 females and 3 males (Cecere et al. 2003). *Panstrongylus megistus* and *T. dimidiata* reach much lower densities than *T. infestans* or *R. prolixus*. The greater nutritional value of human blood adds an unappreciated advantage to domesticity (see Sect. 2.5).

Domesticity carries with it several implications. The stability/instability habitat divide connects with the large/small population size dichotomy, whether bug population dynamics are density-dependent or density-independent (and dominated by stochastic events such as weather, host death, and exposure to predators) and modify the effectiveness of vector control efforts (Sect. 2.6 and 2.7).

2.3 Habitat Use and Quality for Triatomines

A unified definition of habitat includes "the resources and conditions present in an area that produce occupancy—including survival and reproduction—by a given organism (Hall et al. 1997)." These include physical factors (such as temperature and moisture) and biotic factors (such as the availability of food and shelter and the absence of toxins or predators). This and other definitions of habitat supersede the notion that it merely represents physical space. Habitat use is considered selective if it is used disproportionately to its availability or, more in point, to its accessibility. Accessibility depends on interspecific interactions as well as social and environmental factors that might limit access to a given resource (Beyer et al. 2010). Although many triatomine species have characteristic habitat types with associated host species, the mechanisms underlying the observed habitat selection patterns remain mostly unknown.

Each triatomine species selects refuges for optimal conditions of temperature, relative humidity, and darkness at the microhabitat level (Lorenzo and Lazzari 1999; Lazzari et al. 2013) and marks suitable refuges via assembling factors present in their feces (Lorenzo and Lazzari 1996). These factors induced aggregation in nymphs of *T. sordida* and *T. infestans*, thus acting as a pheromone and kairomone (Lorenzo Figueiras and Lazzari 1998). Detailed site-level surveys before and after community-wide insecticide spraying in two rural areas of the Argentine Chaco have shown that sympatric triatomine species tended to occupy distinct peridomestic habitats (e.g., *Triatoma garciabesi* versus *T. guasayana* or *T. infestans*) or were positively associated both at site and house level (*T. sordida* and *T. infestans*) (Rodríguez-Planes et al. 2016, 2018). However, none of the secondary species were able to establish colonies and persist in domestic premises after the quasi-elimination of *T. infestans* despite recurrent invasion events (e.g., Rojas de Arias et al. 2012; Rodríguez-Planes et al. 2020).

Most assessments of habitat use have been derived from the frequency distribution of occurrence or relative abundance of triatomine bugs in field surveys. This information was used to rank the suitability of multiple habitats for a given species and to identify which of them may function as key sources of triatomines for house reinfestation after control interventions. For example, initial assessments of habitat

suitability for *T. infestans* in the Argentine Chaco were accomplished via surveys of habitat-specific infestation and bug abundance. These surveys suggested that chicken coops, goat corrals, and pig corrals were the most important ecotopes (López et al. 1999; Cecere et al. 2004, 2006, 2013; Gürtler et al. 2004; Ceballos et al. 2005). However, a broader survey measuring several fitness-related components in more habitat types showed that chicken coops and human sleeping quarters were relatively more important while goat corrals were at the other extreme (Gürtler et al. 2014b). These fitness components (sex ratio, female fecundity, nutritional status, and vector abundance) were in theory better descriptors of habitat suitability. Bug abundance and fecundity were aggregated across ecotopes, suggesting that bug population growth was concentrated in a few productive, high-quality sites.

These patterns are consistent with the 80-20 rule, by which approximately 80% of the vectors or disease cases would occur in 20% of the sample sites (Woolhouse et al. 1997). Using a different theory and methods, Taylor's law of fluctuation scaling described accurately the mean and variance of the habitat-specific abundances of four triatomine species in the Argentine Chaco, with slopes indicating spatial aggregation or variation in habitat suitability (Cohen et al. 2017). Both spatial aggregation and variation in habitat suitability are relevant for improved vector control and surveillance. Extending these approaches to other triatomine species and settings may provide new perspectives on their high-quality habitats.

One dimension of habitat quality relates to physical structure. Housing design and construction materials combined with their degree of maintenance determine the availability of refuges for domestic bugs, such as cracked walls, tiled or thatched roofing, and earthen floors (Dumonteil et al. 2013; Bustamante et al. 2014), affect the susceptibility of a house to bug invasion and subsequent colonization (Monroy et al. 2009), and may modify the effectiveness of insecticide applications (Gürtler et al. 2004; Cecere et al. 2013). The surface structure of indoor walls (unplastered or with many crevices) and the existence of thatched roofs were significant predictors of domestic infestation and abundance of the main domestic vectors of *T. cruzi* (Cecere et al. 1998; Mott et al. 1978a; Rojas de Arias et al. 1999; Andrade et al. 1995).

Experimental evidence showed that bug population size increased steadily with refuge availability over a 2-year period, with two out of three bug populations in low-refuge huts becoming extinct (Cecere et al. 2003). However, the exact way in which housing quality affects domestic infestation may vary between triatomine species. Dirt floors are highly important for *T. dimidiata* as it uses dirt for camouflage (Zeledón and Vargas 1984; Bustamante et al. 2014) but appear to be of little utility to *T. infestans*, which lacks a camouflage behavior. Dirt floors are indicators of high social vulnerability and frequently are associated with other household features favoring house infestation and triatomine abundance, e.g., mud walls and overcrowding (Fernández et al. 2019a). Window screens greatly reduced the domestic invasion of *T. dimidiata* in Yucatan, Mexico (Waleckx et al. 2015b). Peridomestic structures also provide numerous shelters, as in chicken houses and animal enclosures built with mud, wood, rock, or piled thorny shrubs (Carcavallo et al. 1999; Cecere et al. 2004; Walter et al. 2007; López et al. 1999; Diotaiuti et al. 2000; Gurevitz et al. 2013; Ramsey et al. 2003). If bug-proof chicken houses replaced the

current structures used in northeast Argentina, *T. infestans* and *T. sordida* would lose a key productive habitat (Gurevitz et al. 2013; Rodriguez-Planes et al. 2018). In general, peridomestic structures function as host and triatomine breeding sites and serve as sources of insects that invade human habitations (Cecere et al. 2004, 2006).

2.4 Host Availability

Host availability is quantified by the local abundance of avian or mammalian hosts. However, a host may be available but not accessible if host-seeking triatomines cannot actually feed on it because of some actual or virtual barrier (e.g., bednets, repellents, distance). In domestic habitats, the most frequent hosts of triatomine bugs are humans, dogs, cats, chickens, and rodents. Domestic bug abundance increased with increasing numbers of human residents in rural communities infested with *T. infestans*, *P. megistus*, *R. prolixus*, or *T. dimidiata* (Marsden et al. 1982; Piesman et al. 1983; Gurevitz et al. 2011; Campbell-Lendrum et al. 2007). Moreover, the presence of indoor-resting chickens and dogs correlated positively with domestic bug abundance across settings and species (e.g., Lardeux et al. 2015; Dumonteil et al. 2013; Bustamante et al. 2009, 2014; Cecere et al. 1998; Gurevitz et al. 2011). These relationships tend to be consistent with the outcome of domestic triatomines' blood meal surveys (Sect. 2.5).

Host demography and domestic animal management practices add another dimension to the links between host availability/accessibility, vector abundance, and socioeconomic position, as the habits of keeping chickens indoors for protection and letting domestic dogs wander freely or share domestic premises are frequent in rural areas under a subsistence economy. For example, in typical rural villages of the Argentine Chaco, seasonal breeding pulses determine a surge of host numbers with the appearance of juveniles and their enhanced exposure to triatomines. The occurrence of indoor-nesting chickens peaked in spring and decreased during the hot summer months toward reaching a minimum over fall-winter (Cecere et al. 1997). Permanent chicken coops were rare and nesting sites relatively instable over time; in the absence of the host, the starved bugs presumably dispersed and generated new foci. The household number of chickens varied widely over time depending on household acquisition, consumption, and disease outbreaks (Rodríguez-Planes et al. 2018). In goat corrals, kids kept enclosed for protection were continually exposed to triatomines, while adult goats were allowed to roam and forage the little grass available in the dry Chaco (Ceballos et al. 2005). The population size of domestic dogs remained stable from year to year though with high turnover rates (Gürtler et al. 1990). These fine-scale spatial and temporal heterogeneities affect triatomine vital rates and population size.

Host behavior patterns also affect host accessibility. Although household size varies little from year to year, in practice householders exposed to hot weather or nuisance pests move their beds and hammocks to open air as an adaptive response (see Sect. 2.5) and thus may become less accessible to domestic triatomines

(Rabinovich 1985; Gürtler et al. 1997; Brenière et al. 2017). In doing so, however, householders may become more exposed to peridomestic or sylvatic vectors such as *R. pallescens*. Households also differed in letting domestic dogs and cats roam freely for all or part of their food. Dogs have a marked crepuscular free-ranging behavior, and male dogs wander in small packs at night during estrus periods (Matter and Daniels 2000). In rural areas throughout Latin America, it is quite common that dogs are not neutered. These host activity patterns may reduce the likelihood of host-vector encounters. Thus, scoring the actual presence or absence of domestic hosts (past and current) presents special challenges. Site-level occupancies may vary widely over time depending on weather, host behavior, biting insects, and cultural patterns. Triatomines may respond fast to the sudden absence of a host and actively disperse (Castillo-Neyra et al. 2015).

2.5 Blood-Feeding Performance

Host-Feeding Patterns The host-feeding patterns of Triatominae are key to understanding the eco-epidemiology of Chagas disease. Most species of Triatominae show eclectic blood-feeding patterns on birds and mammals (Wisnivesky-Colli 1987; Rabinovich et al. 2011), with very few triatomine species displaying a remarkable specificity for a host species (e.g., *C. pilosa* for bats in caves) (Dujardin and Schofield 2004). Some triatomine species may occasionally feed on reptiles, on amphibians, and on insect hemolymph. The small nymphs may even feed on blood-engorged conspecifics (cleptohematophagy). In domestic habitats, the main blood meal sources of triatomine bugs are humans, dogs, chickens, and cats. House mice and rats are also a relevant blood meal source for domestic populations of *T. dimidiata* and *Triatoma barberi* (De Urioste-Stone et al. 2015).

The species of Triatominae with significant public health relevance are those that feed on humans or which may contaminate foodstuffs. Table 1 identifies 24 species of Triatominae with documented evidence of having fed on humans as determined by immunologic methods, estimated from the data compiled by the most recent review on triatomine blood-feeding patterns (Rabinovich et al. 2011). From the 159 data sets, we selected those that included any species collected in domestic habitats only, or in domestic or peridomestic habitats only, and excluded all other habitat sources. The selected list includes 68 studies and 28,054 specimens tested.

The degree of human-vector contact, measured by the average human blood index (i.e., the fraction of tested insects having a human blood meal), peaked in *R. prolixus* (79.2%), *R. pallescens* (65.0%), *T. pallidipennis* (44%), *T. infestans* (40.3%), and *P. megistus* (36.6%). A second group of species had a mean human index between 10% and 20% (*P. herreri*, *T. barberi*, *R. pictipes*, *T. vitticeps*, *R. ecuadoriensis*, *T. dimidiata*, *T. brasiliensis*, and *T. pseudomaculata*). Chickens ranked high, followed by dogs and cats. The rodent blood index averaged 17.1% across all species in the list and frequently exceeded 40%. Even sylvatic populations of

Table 1 Host-feeding patterns of Triatominae collected in domestic or peridomestic habitats

Species	No. of studies	No. of insects tested	Human blood index		Dog blood index		Cat blood index		Chicken blood index		Rodent blood index	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
R. prolixus	6	517	79.2	18.8	6.3	4.8	9.3	19.1	3.3	5.3	0.3	0.8
R. pallescens	2	1391	65.0	18.4	1.0	1.4	0.5	0.7	9.0	7.1	6.0	4.2
T. pallidipennis	1	18	44.0	6.0			0.0	0.0			6.0	
T. infestans	11	5134	40.3	20.5	11.9	11.1	9.0	5.0	22.2	10.1	4.6	8.7
P. megistus	7	5728	36.6	31.7	4.9	1.5	3.9	6.5	30.7	20.0	5.3	6.2
P. herreri	1	77	21.0	8.0			1.0	16.0			55.0	
T. barberi	2	958	20.5	3.5	7.0	1.4	7.0	1.4	10.0	0.0	47.5	3.5
R. pictipes	1	10	20.0	20.0			10.0	30.0			20.0	
T. vitticeps	1	79	14.0	11.0			0.0	11.0			4.0	
R. ecuadoriensis	1	8	13.0	0.0			0.0	50.0			25.0	
T. dimidiata	5	933	11.2	8.1	13.0	11.3	1.0	1.4	23.0	23.4	39.8	44.1
T. brasiliensis	2	1196	11.0	15.6	3.0	4.2	4.5	6.4	75.0	25.5	1.5	2.1
T. pseudomaculata	2	3789	10.5	13.4	7.5	9.2	2.0	0.0	59.0	22.6	12.0	15.6
T. rubrofasciata	2	548	9.5	4.9	22.5	0.7	0.5	0.7	3.5	2.1	47.5	2.1
T. rubrovaria	2	153	6.0	4.2	0.0	0.0	0.0	0.0	8.0	11.3	6.5	4.9
T. sordida	14	6703	5.4	6.0	5.1	6.7	0.6	1.1	58.5	19.7	12.5	7.2
P. lutzi	1	79	4.0	0.0			1.0	32.0			28.0	
R. nasutus	1	135	4.0	2.0			0.0	93.0			0.0	
T. maculata	1	52	4.0	0.0			8.0	73.0			15.0	
B. ferroae	1	121	1.0	3.0			0.0	0.0			4.0	
R. neglectus	1	181	1.0	2.0			0.0	75.0			13.0	
P. herreri	1	76	0.0	0.0			0.0	0.0			0.0	
P. geniculatus	1	2	0.0	50.0			0.0	50.0			0.0	
T. costalimai	1	166	0.0	0.0			0.0	16.0			58.0	

Summary estimates for humans and domestic or synanthropic animals from data compiled by Rabinovich et al. (2011)

T. infestans had human blood meals (Buitrago et al. 2013, 2016), as did sylvatic *Mepraia spinolai* (Brenière et al. 2017) and *T. sanguisuga* (Waleckx et al. 2014). Many other species have been reported to attack humans in the wilderness, such as *R. brethesi*, *T. guasayana*, and *T. brasiliensis*. In summary (i) many triatomine species other than the main domestic vectors blood-feed on or may attack humans under the appropriate circumstances; (ii) the rates of human-vector contact largely differed between and within species, habitats, and settings; (iii) the recorded patterns are fraught with a putative selection bias, as there is sparse information for the great majority of triatomine species; and (iv) mixing domestic and peridomestic bug collections deflates human blood indices and increases chicken blood indices, for example. The fact that triatomine host-feeding patterns are seldom related to local host numbers (i.e., at site or house level), host, and vector infection hampers the full understanding of system dynamics.

The host-feeding choices of hematophagous insect vectors are affected by the host species composition in a given habitat, relative host abundance, host proximity, and host defensive behavior (Lehane 2005). Host proximity has usually been considered more important than any intrinsic host preference for a host-seeking bug (Minter 1976). However, there were very few controlled host choice experiments in Triatominae. For *T. sordida* (typically associated with birds), first-instar nymphs significantly preferred birds to humans (Rocha e Silva et al. 1977), whereas the feeding success and blood meal size of fifth-instar nymphs were significantly larger on guinea pigs than on pigeons (Crocco and Catalá 1997). In laboratory-based trials, four caged vertebrate host species were simultaneously exposed to separate groups of fifth-instar nymphs of *T. infestans*, *T. dimidiata*, and *R. prolixus* (Jirón and Zeledón 1982); none of these species displayed definite host-feeding preferences among dogs, chickens, and opossums, but toads were rarely selected. Dogs were highly preferred over chickens or cats in host choice experiments conducted in small huts where the released bugs could choose to feed on any of the two host species available (Gürtler et al. 2009). Bugs that fed on dog engorged significantly more than bugs that fed on chicken or cat, suggesting dogs were more tolerant to bites than chickens or cats. These results were mainly consistent with field host-feeding patterns.

Host defensive behavior in response to triatomine bites determines the selective utilization of some host species and individuals (Kelly and Thompson 2000). Several triatomine species displayed negative density-dependent engorgement rates on non-anesthetized, unrestrained small chickens, pigeons, and rodents in laboratory settings (Crocco and Catalá 1997; Rabinovich 1985; Piesman et al. 1983; Schofield 1982). In the host choice trial described above, high vector densities tended to increase the shifts between hosts and significantly increased post-exposure bug weight though not to a large extent (Gürtler et al. 2009).

These basic processes combined with the rather limited dispersal range of Triatominae (especially of nymphs) determine that their host-feeding patterns tend to be spatially structured according to habitat type and correlate closely with the main local resident host(s). In rural villages in northwestern Argentina, the main or only blood meal sources of *T. infestans* in chicken coops, goat corrals, and pig

corrals were chickens, goats, and pigs, respectively (Gürtler et al. 2014b). The domestic populations of *T. infestans* displayed seasonal variations in feeding patterns related to the shifting resting sites of hosts during the hot season (Gürtler et al. 1997). In spring-summer domestic bug collections, the human blood index decreased substantially with the increasing presence of chickens or dogs indoors and also decreased with increasing domestic bug abundance, reflecting changes in human exposure. Moreover, the dog blood index increased significantly with increasing numbers of dogs and with domestic bug abundance and decreased as the chicken blood index rose. The presence of indoor-resting chickens correlated positively with domestic bug abundance and with an increasing chicken blood index (Cecere et al. 1997). Underlying these patterns are the opportunistic nature of host choice (expressed in high rates of mixed blood meals on different host species) and the spatiotemporal variations in host availability and accessibility. The inverse relationship between the human and chicken blood indices in domestic *T. infestans* was verified in at least two other rural settings in northern Argentina (Gürtler et al. 2014a; Ordóñez-Krasnowski et al. 2020). Host shifts affected domestic transmission (see Sect. 3.6).

Host Blood and Fitness The question on whether host blood type affects the fitness of triatomines has implications for understanding domesticity, population growth, and transmission risks. Host blood effects are well known in mosquitoes and tsetse flies (Lehane 2005) and affected the vital rates and engorgement levels of triatomines (Gardiner and Maddrell 1972; Núñez and Segura 1987). Human blood was better than sheep blood for vitellogenesis, general metabolism, and development in *R. prolixus* (Valle et al. 1987). Cohorts of *R. prolixus* that fed artificially on citrated blood during their entire lives in the same artificial environment achieved faster development rates and much larger blood meal size, body weight, and female fecundity when fed on human or rabbit blood rather than when bugs fed on chicken, sheep, or horse blood (Gomes et al. 1990). Avian blood usually has much lower hemoglobin and plasma protein than the blood of clinically healthy mammals and hence appears to be nutritionally inferior. However, the lower blood viscosity of avian blood allows increased ingestion rates and would reduce the risk of host-induced death (Lehane 2005). Goat (or sheep or cow) blood reduced the feeding efficiency of *T. brasiliensis* because triatomines are unable to agglutinate cattle erythrocytes (Araujo et al. 2009). Gardiner and Maddrell (1972) reported that *R. prolixus* bugs tended to blood-feed much less or not at all on adult goats or sheep that had been exposed repeatedly to bites (i.e., developed acquired resistance) and hence laid fewer eggs. This suggested that the rather large field populations of *T. infestans* in goat corrals most likely thrived at the expense of the immunologically naïve kids (Gürtler et al. 2004, 2017). When suitable hosts are not available, goat corrals become sources of dispersing triatomines as their nutritional state declines.

Blood-Feeding Rate and Blood meal Size These parameters affect the vital rates of triatomine bugs, including their propensity to initiate flight, the regulation of local population size (Schofield 1994), and parasite transmission rates. There are

few estimates of the blood-feeding rates, blood meal sizes, and nutritional status of Triatominae in field settings (reviewed in Gürtler et al. 2014b). Preliminary estimates of the feeding rates of domestic *T. infestans* and *R. prolixus* based on the distributions of body weight and body length yielded 5–8 to 5–20 days, respectively (Schofield 1980; Rabinovich et al. 1979). By measuring the temperature-adjusted occurrence of transparent urine assessed shortly after capture in experimental chicken coops, the blood-feeding rate of *T. infestans* proved to be temperature- and bug density-dependent (Catalá 1994). Domestic populations of *T. infestans* blood-fed every 3–4 days over the spring-summer period (Catalá et al. 1997; Gürtler et al. 2014a). Mean feeding intervals varied widely across ecotopes and peaked in chicken coops (López et al. 1999; Ceballos et al. 2005; Gürtler et al. 2014b). Some triatomine species may feed every 2 days in the insectary and withstand prolonged starvation over several months.

The only field estimate of blood meal size comes from experimental chicken houses stationed outdoors (Catalá 1994). The total blood meal contents of recently fed triatomines may be used as a proxy of blood meal size under assumed steady-state conditions (Gürtler et al. 2017). Human- and chicken-fed *T. infestans* had significantly larger blood contents than bugs fed on other hosts, whereas goat-fed bugs ranked last, in consistency with their average blood-feeding rates. Female fecundity was also maximal in chicken-fed bugs from chicken coops and minimal in goat-fed bugs. The greater blood-feeding performance and nutritional status of bugs from chicken coops, closely followed by domestic bugs, reflected in their having a larger body length than other peridomestic bugs across most life stages – a clear indication of habitat-associated fitness advantages.

Timing of Defecation Blood meal size determines the timing of defecation and hence the chance of eventual skin contamination with triatomine feces (Kirk and Schofield 1987; Trumper and Gorla 1991). Blood meal size and the timing of defecation were both inversely density-dependent. As host irritation increases, its defensive reactions lead to interrupted (smaller) blood meals and prolong the time to the first fecal drop. These mechanisms supported the hypothesis that the greatest chances of transmission of *T. cruzi* would occur at low bug densities. However, it does not necessarily follow from the above experiments that a feeding contact with a single bug at high bug population density has a lower risk of transmitting infection to an uninfected mammalian host than a feeding contact with a single bug at a low bug population density (Cohen and Gürtler 2001). The time needed to secure a replete blood meal and the timing of defecation differed substantially among the main triatomine vectors (Zeledón et al. 1977); both *T. infestans* and *R. prolixus* defecated during or shortly after blood-feeding while on the host. Although the North American species of Triatominae were once considered poor vectors based on their long defecation times, subsequent studies revealed that several of them would be efficient vectors (Zeledón et al. 2012).

2.6 Environmental Variables

Temperature and precipitation affect the geographic distribution of Triatominae (Gorla and Noireau 2017), their host-seeking and metabolic rates, body size, and vital rates. Before the onset of large-scale insecticide spraying campaigns in Argentina during the 1960s, house infestation with *T. infestans* occurred in areas where daily maximum temperatures exceeded 20 °C and vapor-pressure deficits were greater than 1100 hectopascals over at least 220 days a year (Curto de Casas et al. 1999). The minimum temperature of the coldest month apparently defined the geographic range of *T. infestans* and *R. prolixus*, the latter being also influenced by precipitation-related variables (Medone et al. 2015). At finer scales, triatomines are affected by variable microsite conditions. Different species display specific patterns of thermopreference and hygropreference that vary substantially over the feeding and daily cycles and affect refuge selection (Lazzari et al. 2013). For example, both egg hatching and molting success are severely reduced by very low relative humidity.

Environmental variables interact with habitat structure to generate heterogeneous microsite conditions. The domestic and peridomestic habitats of *T. infestans* damped external temperatures to different extents depending on their physical structure, ranging from a minimum in thorn shrub-fenced goat corrals to a maximum in typical mud-and-thatch human habitations (Vazquez-Prokopec et al. 2002). In general, bugs from animal enclosures with little capacity to dampen climatic extremes are exposed to increased risks of hyperthermia and desiccation, which likely trigger dispersal. Large swings in diurnal temperature prolong insect development times, reduce survival and female fecundity relative to constant temperatures (Nijhout et al. 2014), and reduce body size when temperatures reach stressful levels (Colinet et al. 2015). In contrast, goat corrals with thick fences made of piled shrubs and goat dung have a large damping capacity and are able to sustain large triatomine populations despite harsh winters (Schofield 1985). Thus, the exact physical structure of the habitat rather than its main function or host determine whether local conditions are suitable for triatomines.

2.7 Population Dynamics and Vital Rates

Nearly all the available information on triatomine survival, fecundity, and development rates comes from laboratory-reared cohorts held under optimal, constant conditions (e.g., Rabinovich 1972; Perlowagora-Szumlewicz 1975; Rabinovich and Feliciangeli 2015). Triatomine populations are stage-structured and have long life cycles (from 4–6 to 12 months or more). The most realistic approximations to field settings were provided by experimental bug populations kept in huts that mimicked typical rural houses exposed to the temperate climate of central Argentina. The huts were caged with mosquito netting to prevent in- or out-migration and housed either two or four chickens in structurally homogeneous huts (Gorla and Schofield 1989;

Gorla 1991) or one chicken under a three-level gradient of refuges (Cecere et al. 2003).

In these experimental systems, the population abundance of *T. infestans* fluctuated seasonally following temperature variations and peaked once in early- or mid-summer, as in rural houses from Brazil (Dias 1955; Schofield 1980). These populations displayed two peaks of adult emergence of differing intensity (Cecere et al. 2003; Gorla 1991) and peak numbers of eggs per female lagging by 1 month. During spring-summer, fecundity averaged approximately three eggs per female per day in huts with two to four chickens (Gorla and Schofield 1989) and in huts with maximum refuge availability and one chicken (Cecere et al. 2003). These estimates are lower than the average fecundity of *T. infestans* (4.07 eggs per female per day) fed twice a week and kept under optimal conditions (Núñez and Segura 1987). Both female fecundity and nymphal development rates steadily increased with increasing temperature above 16 °C (the threshold for development), whereas mortality was mainly determined by monthly mean minimum temperatures (Gorla 1992). A stage-structured stochastic model of the population dynamics of *T. infestans* described well the temperature-dependent seasonal variations in bug abundance, stage structure, and the two peaks of female fecundity rates and total egg numbers observed in hut experiments (Castañera et al. 2003).

In the experimental huts, the egg-to-adult mortality of *T. infestans* ranged from ~98.5% (Gorla and Schofield 1989) to 94.8–97.6% (Cecere et al. 2003) and exceeded a preliminary estimate for domestic *T. infestans* (86.3%) based on the recovery of dead bugs and exuviae (Schofield 1980). In one of the studies (Cecere et al. 2003), a founder female had a maximum life span of 20 months, and the average for adult males (7.8) and females (5.6) exceeded other estimates of 4–5 months (Gorla and Schofield 1989). Bug mortality was density-independent, and female fecundity was weakly density-dependent (Gorla and Schofield 1989; Gorla 1991). Domestic populations of *R. prolixus* also failed to display density-dependent mortality (Rabinovich 1985). Chickens most likely were a refuge-dependent mortality factor: both nymphs and adults from huts with plastered walls had lower survival rates than bugs from maximum-refuge huts (Cecere et al. 2003). In the absence of migration, the net reproductive rate (R_0) decreased steadily from 3.91 to 0.25 in maximum- to minimum-refuge huts, respectively (Cecere et al. 2003). Schofield (1980) estimated $R_0 = 8.7$ by combining laboratory and field data for domestic *T. infestans*. Rabinovich (1972) estimated $R_0 = 25.04$ for laboratory-reared cohorts of *T. infestans* having very large survivorship and very low fecundity. These estimates of R_0 reflect the species' large capacity for establishing new foci and for population recovery following insecticide treatment.

Insecticides are a major source of bug mortality even when they are applied by householders. In the absence of government-sponsored insecticide campaigns, domestic bug abundance was negatively related to the domestic application of insecticides carried out by householders (Gurevitz et al. 2011; Gaspe et al. 2015). With increasing access to insecticides, especially in agricultural settings, the population size of domestic triatomines may rarely reach the typical high levels recorded in the past at which density-dependent effects on vital rates may set in. Rather,

domestic bug populations may be held at the lower limits imposed by insecticide use, housing improvements, and environmental stochasticity. These smaller bug populations occupying discrete patches connected by frequent active dispersal (zu Dohna et al. 2009), subject to stochastic events, may dominate the current and future eco-epidemiological scene.

Dispersal plays a key role in the invasion of domestic premises and establishment of new colonies, the recovery of bug population size after control interventions, and the spatial structure of triatomine populations. It may also contribute to the regulation of local population size, as suggested by the density-dependent loss of marked adult *R. prolixus* from domestic premises (Rabinovich 1985). In contrast, flight initiation was inversely related to *T. infestans* density in a laboratory setting (McEwen et al. 1993), suggesting that other processes may be implicated (e.g., pheromone-mediated searches for mates).

The dispersal of triatomine bugs is accomplished by active (walking, flight) or passive means via carriage in clothes, luggage, and firewood or as eggs stuck to the feathers of some birds (Schofield 1994). Passive transport of domestic triatomines is always a possibility, as shown by the finding of a *T. infestans* adult further south of its historical geographic range (Piccinali et al. 2010). Flight dispersal is limited to a few 100 m up to a few km, whereas passive dispersal has been associated with the long-distance range expansion of *T. infestans* to northeast Brazil, *R. prolixus* to Central America, and *T. dimidiata* to Ecuador. Mark-recapture studies demonstrated an intense exchange of nymphs and adults of domestic *R. prolixus* between houses located 100 and 500 m apart, consistent with its well-known flight capacity (Rabinovich 1985). The flight range of *T. infestans* (considered a poor flier) may exceed 2400 m, as suggested by sustained tethered flights at speeds of 2 m/s for at least 20 min (Ward and Baker 1982). Walking dispersal of nymphs and adult bugs may play a substantial role in establishing new foci at finer scales and contribute to the spatial aggregation of infestation (Vazquez-Prokopec et al. 2006; Abrahan et al. 2011). It may also explain the finding of human-fed triatomines in nearby peridomestic habitats not used as human resting sites (Cecere et al. 1997; Gürtler et al. 2014b).

Flight dispersal invariably includes unfed triatomines and occurs within the first 2–3 h after sunset in most of the species investigated (Di Iorio and Gürtler 2017). The initiation of flights is usually triggered by starving conditions (reflected in low body weight-to-body length ratios) and mean temperatures in the range from 20 to 30 °C. The duration and detailed time structure of the dispersal season are relevant because they determine subsequent establishment events and eventual human exposures. Analysis of a long time series of site infestations in a rural village suggested increased dispersal of *T. infestans* during spring and a 6-month lag between a new bug establishment on a site and dispersal from this site (zu Dohna et al. 2009).

Artificial light sources usually attract adult triatomines of many species and favor house invasion. Public streetlights were positively and significantly associated with domestic infestation with *T. dimidiata* in Yucatan, Mexico (Pacheco-Tucuch et al. 2012). When the streetlight posts of a small village in western Argentina were systematically inspected for triatomines between sunset and midnight over

spring-summer, the occurrence of flight-dispersing triatomines (from four species) steadily increased between 16.6 and 31.7 °C, suggesting a putative temperature threshold for flight initiation at 17–18 °C (Di Iorio and Gürtler 2017). Although the catch of adult *T. infestans* at the streetlight posts was sex-independent, that of *T. guasayana* was strongly male-biased – a pattern that has been recorded elsewhere and in other triatomine species. In an experimental setting, male *R. prolixus* bugs increased substantially their takeoff activity in response to female pheromones, but the reverse did not occur (Zacharías et al. 2010). This suggests that a colonizing, unfertilized female triatomine may be able to recruit flight-dispersing males and thus increase the chances of establishing a viable bug colony. However, flight dispersal may also be female-biased, as suggested by field observations of individually marked adult *P. megistus* (Forattini et al. 1977) or *T. infestans* in open chicken coops and by microsatellite-based genetic studies (reviewed in Gürtler et al. 2014b). Sex-biased flight dispersal may contribute to imbalanced adult sex ratios across ecotopes (Payet et al. 2009).

3 Biological and Ecological Factors Related to Parasite Transmission

3.1 Parasite Diversity

Trypanosoma cruzi (Kinetoplastida: Trypanosomatidae) has a genetically diverse clonal structure classified into six genotypes (TcI-TcVI) denominated discrete typing units, or DTUs (Zingales et al. 2012). Whether TcBat, a recently described genotype mostly restricted to bats, is a different DTU is still under debate (Marcili et al. 2009; Zingales 2018). All DTUs are capable of infecting humans and mammals, and their geographical distribution and frequency of occurrence vary widely across the Americas (reviewed by Brenière et al. 2016). Humans and > 150 species of nonhuman mammalian hosts have been found naturally infected with *T. cruzi*; other vertebrates are refractory to the infection (Jansen et al. 2017).

Transmission Cycles Domestic and sylvatic habitats sustain two main types of transmission cycles (Miles et al. 2003). Sylvatic cycles involve sylvatic mammals and sylvatic triatomine species, whereas domestic cycles mainly include domestic triatomines, humans, and domestic or synanthropic animals. The two archetypical sylvatic cycles across the Americas are an arboreal cycle involving didelphid marsupials and TcI and a terrestrial cycle involving armadillos and TcIII (Yeo et al. 2005; Brenière et al. 2016). However, these associations are not absolute. TcI has the broadest distribution from southern United States to Argentina and Chile. It has been mainly found in sylvatic cycles across the Americas and in domestic cycles to the north of the Amazon basin. TcII has been mainly associated with domestic cycles but has also been isolated from sylvatic mammals. TcV and TcVI predominate

in domestic cycles across the Southern Cone countries. TcBat, TcIII, and TcIV have been mainly found in sylvatic cycles.

The degree of connectivity or overlap between domestic and sylvatic cycles may affect disease control and elimination efforts. If transmission cycles overlap, the introduction of sylvatic parasites may threaten efforts directed at curtailing domestic transmission, as recorded in Mexico (Ramsey et al. 2012). Overlapping transmission cycles of TcI and TcVI were also recorded in Yucatan, Mexico (López-Cancino et al. 2015), Guatemala (Pennington et al. 2015), and Venezuela, where *Rhodnius* bugs infested both houses and palm trees (Miles et al. 2003). A classic example of separate transmission cycles occurred in Bahia, Brazil, where TcII circulated in houses infested with *P. megistus* while TcI circulated between *T. tibiamaculata* and *Didelphis albiventris* opossums in bromeliad epiphytes (Miles et al. 2003). Separate transmission cycles occurred in the Argentine Chaco region, where TcV/TcVI predominated in domestic habitats while TcI and TcIII were restricted to sylvatic hosts (Cardinal et al. 2008; Diosque et al. 2003; Macchiaverna et al. 2015, 2018; Orozco et al. 2013; Enriquez et al. 2013; Lucero et al. 2016).

3.2 Domestic Reservoir Hosts

Nonhuman Reservoir Hosts Dogs, cats, rodents, and domesticated guinea pigs are major domestic nonhuman reservoir hosts of *T. cruzi* (reviewed in Gürtler and Cardinal 2015). They are able to maintain *T. cruzi* in the absence of any other host species and play key roles as amplifying hosts and parasite sources in many domestic or peridomestic transmission cycles across ecoregions and triatomine species. House mice and rats contributed to domestic bug infection with *T. cruzi* in many settings (Bustamante et al. 2014; Rosal et al. 2018). Community-based rodent control measures significantly reduced rodent infestations and the prevalence of *T. cruzi* infection in early-stage nymphs of *T. dimidiata* (De Urioste-Stone et al. 2015).

Human Hosts The prevalence of human infection with *T. cruzi* attests to the potential magnitude of the disease and to the size of the human reservoir. Humans constitute a many decade-long reservoir of *T. cruzi* unless an effective treatment is administered. In contrast, other nonhuman domestic reservoir hosts have large turnover rates, which combined with effective vector control actions produce a fast clearance of infected individuals, which are mostly replaced with uninfected ones (Gürtler et al. 1990; Gürtler and Cardinal 2015).

3.3 Human Infection

Humans typically acquire a vector-borne infection with *T. cruzi* while sleeping at night at their usual resting location or during short visits to other villages (Gürtler et al. 2007b). Incidental human infection may sporadically occur in campsites and as an occupational hazard (Brenière et al. 2017). Human exposure may eventually derive from infected triatomines dispersing from peridomestic outhouses (Cardinal et al. 2014) or from the interface with sylvatic habitats, as in the dry-shrub fences harboring *T. eratyrusiformis* in northern Argentina (Cecere et al. 2016), stone piles infested with *T. pallidipennis* in Mexico (Brenière et al. 2017), and palm trees with intrusive *Rhodnius* sp. and other triatomine species (Jácome-Pinilla et al. 2015; Abad-Franch et al. 2015). Human susceptibility to *T. cruzi* infection appears to be independent of age and gender.

The infection involves a short acute phase and a lifelong chronic phase and is irreversible unless the patient is treated with nifurtimox or benznidazole. A very small fraction of *T. cruzi*-seropositive individuals spontaneously revert to a seronegative status in the absence of etiologic treatment (e.g., Morillo et al. 2015). The death rate of untreated individuals during the acute phase ranged from 2 to 12% and was inversely related to patient age (Dias and Schofield 2017), as does the chance of a symptomatic presentation (Romaña 1963). A variable fraction (20–30%) of the survivors develop cardiac disease and suffer increased death rates between 35 and 50 years of age, especially males.

The bug-to-human transmission probability (b) is the probability that, in one feeding contact between one infected triatomine and an uninfected human, the human acquires a *T. cruzi* infection. Using indirect methods, b was estimated to range between 0.00026 and 0.0011 (Rabinovich et al. 1990; Nouvellet et al. 2013). Combined with vectorial capacity and other variables, b was used to estimate the threshold density of infected vectors required to initiate transmission chains and allow the infection to persist in the community. Vector control programs have extrapolated this rationale to the notion of a threshold domestic infestation prevalence associated with vector-borne transmission (Aiga et al. 2012). In practice, measuring each of the variables involved in vectorial capacity with any accuracy is fraught with major difficulties (Dye 1992). Several studies revealed the occurrence of prevalent or incident human cases at very low densities of *T. cruzi*-infected domestic triatomines per unit of search effort (Piesman et al. 1985; Rabinovich et al. 1990; Gürtler et al. 2005; Cardinal et al. 2018). Furthermore, the relation between bug population density and the probability of transmission by contamination during a single feeding contact with an infected bug is hard to assess empirically. Estimation of a threshold density of infected bugs for domestic transmission, if there is any, is additionally hindered by the low sensitivity and imprecision of triatomine sampling methods (Abad-Franch et al. 2014; Rojas de Arias et al. 2012) and human mobility (see Sect. 4.3).

3.4 Host Infectiousness

This key parameter has usually been measured by xenodiagnosis in at least three partially related forms, including the proportion of uninfected vectors that become infected after a replete blood meal on an infected host (i.e., host infectiousness), and more lately by real-time PCR (Gürtler and Cardinal 2015). Dog and cat infectiousness determined by xenodiagnosis correlated closely with the concentration of *T. cruzi* DNA determined by quantitative real-time PCR (Enriquez et al. 2014).

Before the implementation of large-scale control campaigns in southeast Brazil, the prevalence of xenodiagnosis-positive dogs (28.6%) and cats (19.7%) largely exceeded that recorded in humans (5.7%) (Freitas 1950). A similar ranking was often recorded in areas infested with *R. prolixus*, *T. dimidiata*, and *T. infestans* (Gürtler et al. 1996; Pifano 1973; Zeledón et al. 1975). Nearly all the triatomines that fed on human acute cases usually became infected regardless of the species or instar used (Minter-Goedbloed et al. 1978), whereas in a review of several studies *T. cruzi*-seropositive patients (presumably in the chronic stage) infected from 2–3% to 26% of xenodiagnosis nymphs (Gürtler et al. 1996). In a recent population-based survey, 60.5% of *T. cruzi*-seropositive humans were infectious to xenodiagnostic triatomines examined by optical microscopy and molecular methods; on average, they infected 5.2% of fourth-instar nymphs, and human infectiousness conformed to the 80-20 rule (Macchiaverna et al. 2020). The infectiousness of *T. cruzi*-seropositive people declined with age (Maguire et al. 1982), unlike the age-independent pattern frequently recorded in dogs from endemic rural areas (Gürtler et al. 1996; Enriquez et al. 2014). Moreover, the mean infectiousness of seropositive dogs to seropositive humans differed by an order of magnitude. Thus, although all mammalian species may be considered potential hosts of *T. cruzi*, their reservoir competence may differ substantially.

3.5 Vector Competence

All nymphal instars and adult stages of Triatominae are susceptible to *T. cruzi*, although perhaps not to the same extent, and may become infected with *T. cruzi* when feeding on an infected mammal carrying bloodstream trypomastigotes. The infection may rarely occur by coprophagy or cannibalism and is mostly irreversible except when the insects are severely starved. Following the infectious blood meal and a short latent period ranging from 2 to 10 days, the intensity of infection increases exponentially up to a maximum reached by 45–60 days postinfection when the bugs are fed regularly on noninfected hosts (Garcia et al. 2007). Parasite multiplication and development of metacyclic trypomastigotes in *T. infestans* was optimal between 23 and 27 °C, nil below 10 °C, and declined at 28 or 36 °C (Neves 1971), anticipating the strong seasonal forcing in human incidence of infection with *T. cruzi* (see Sect. 3.6). The intensity of bug infection varied both with parasite DTU

and triatomine species (Campos et al. 2007; Carvalho-Moreira et al. 2003; de Lana and de Menezes-Machado 2017). The interactions between *T. cruzi* and triatomines are affected by parasite strain, triatomine nutritional state, trypanolytic compounds, digestive enzymes, lectins, gut microbiota, and endocrine system (Garcia et al. 2007; Dumonteil et al. 2018).

The infection with *T. cruzi* reduced the life span of third-, fourth-, and fifth-instar nymphs of *T. infestans* by 14–17% when the insects were severely starved (Schaub 1992), decreased the fecundity and fertility of *R. prolixus*, increased bug mortality in a temperature-dependent way, and prolonged development (Elliot et al. 2015; Marliére et al. 2015; Guarneri and Lorenzo 2017). Whether *T. cruzi* affects the flight dispersal of *T. dimidiata* in a sex-dependent way requires further experimental research (Ramirez-Sierra et al. 2010).

In field settings, the cumulative chances of exposure to an infectious blood meal source are expected to increase over time or with increasing bug developmental stage and blood meal size; these increase the probability of ingesting at least one parasite and the total number of parasites ingested. Bug superinfections (i.e., a new *T. cruzi* infection of an already infected bug) may be quite frequent when the intensity of transmission is high. The prevalence of bug infection increased with bug stage, usually from third instars onward and reached maximum levels in fifth-instar nymphs or adult bugs (e.g., Albarracin-Veizaga et al. 1999; Cardinal et al. 2007, 2014). The intensity of *T. cruzi* infection in *T. infestans* also increased with each successive instar and peaked in late spring, including trypomastigote densities (Giojalas et al. 1990). Only 5–20 to 2000 metacyclic trypomastigotes are needed to experimentally infect a susceptible mouse via the conjunctival, oral, or contaminative cutaneous routes (Eickhoff et al. 2013).

3.6 Transmission Dynamics

Before the implementation of large-scale insecticide spraying campaigns in endemic areas, the seroprevalence of human infection with *T. cruzi* increased nonlinearly with age in stable populations subject to a rather constant risk of infection over time (Fig. 2). Simple catalytic models of pathogen transmission can be fitted to age-specific seroprevalence rates of infection to estimate the per capita rate at which a susceptible host acquired the infection in unit time over the age range covered by the curve (the force of infection) under several assumptions (Muench 1959; Nouvellet et al. 2015). Figure 2 shows the close fit of the irreversible catalytic model to seroprevalence data obtained in two areas of central Argentina in 1956 (Rosenbaum and Cerisola 1961). The force of infection was −0.041 per year (95% confidence interval, −0.0254 and − 0.057) in Ojo del Agua, Santiago del Estero (Fig. 2a), and − 0.045 (−0.053 and − 0.037) in Elcano, Córdoba (Fig. 2b). Seroprevalence rates were predicted, and observed, to reach 100% in age groups >70 years; the 50% infection rates were reached by 20 years of age. More recent surveys also showed that nearly all old-age inhabitants were seropositive for *T. cruzi* in some rural

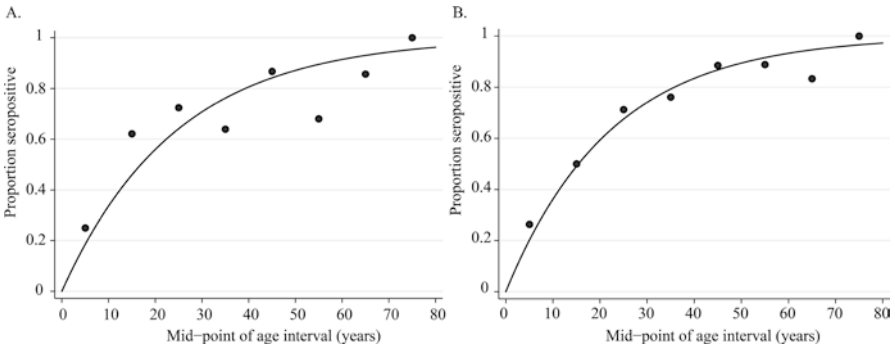

Fig. 2 Observed (dots) and expected (line) age-specific seroprevalence rates for *T. cruzi* infection in humans from Ojo de Agua (**a**) and Sebastián Elcano (**b**), Argentina, May 1956. Data taken from Rosenbaum and Cerisola (1961); seropositivity determined by a complement-fixation test. The line is the fit of the catalytic model with constant force of infection over time and age estimated as in Gürtler et al. (2005)

communities of the Bolivian Chaco (Samuels et al. 2013) and in indigenous communities of Santa Marta, Colombia (Mejía-Jaramillo et al. 2014). At least 50% of those aged >40 years were *T. cruzi* seropositive in the Argentine Chaco (Cardinal et al. 2018; Fernández et al. 2019b).

Before large-scale insecticide campaigns, most *symptomatic* acute cases occurred before reaching 15 years of age, but the distribution displayed a long tail extending up to 52 years of age, with no gender-related asymmetry (Romaña 1963). Figure 3 shows that while the expected seroprevalence of *T. cruzi* infection increased with increasing age in the absence of control actions, the observed frequency of *symptomatic* acute cases declined fast with age, although some occurred even after 50 years of age.

Following the implementation of control actions, the observed seroprevalence rates can be compared with those predicted by other candidate models to infer whether the efforts had effectively diminished the force of infection and by how much (Cardinal et al. 2007; Cucunubá et al. 2017; Feliciangeli et al. 2003; Nouvellet et al. 2015; Samuels et al. 2013).

Seasonality strongly affects the intensity of transmission of *T. cruzi* in subtropical and temperate areas. The incidence of *symptomatic* human cases of Chagas disease displayed similar seasonal variations in northern Argentina across four decades (Romaña 1963; Rebosolán and Terzano 1958; Ledesma Patiño et al. 1992; Lugones et al. 1994). Incidence steeply rose from nonzero minimum values in August (cold season) to peak by November (late spring) and then slightly decreased during the hot summer months (Fig. 4). The relative rate of change in the frequency of acute cases increased faster from late winter to spring than at other times in three of the four data sets. A similar pattern occurred in central Brazil in the 1950s (Schofield 1994). These patterns closely match seasonal variations in temperature acting on triatomine blood-feeding rates (Fig. 5).

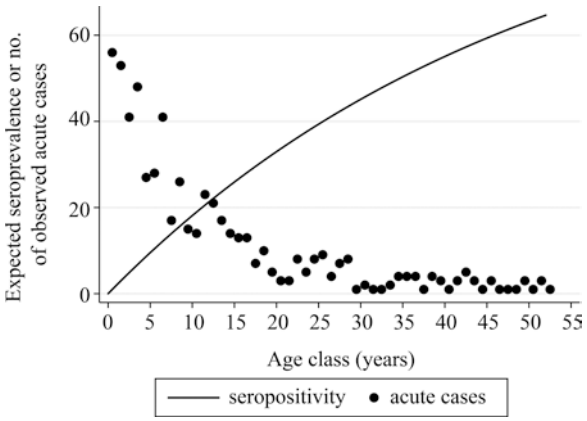

Fig. 3 Expected seroprevalence for *T. cruzi* (estimated through an irreversible catalytic model with force of infection = 0.02 per year, line) and the observed frequency of symptomatic acute cases of human Chagas disease (dots) in Tucumán, Argentina. Data taken from Romaña (1963)

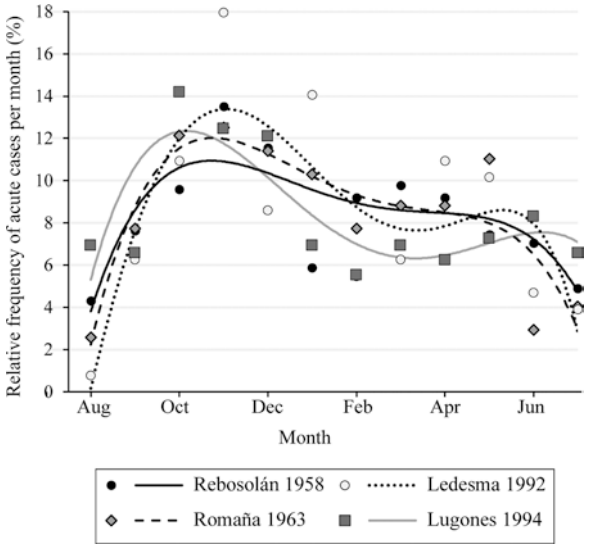

Fig. 4 Monthly-specific relative frequency of symptomatic acute cases of Chagas disease in humans from Tucumán and Santiago del Estero, Argentina. Data taken from Rebosolán and Terzano (1958), including 511 cases diagnosed over 1947–1956; Romaña (1963), including 272 cases; Lugones et al. (1994), including 289 cases, and Ledesma Patiño et al. (1992), including 128 cases over a 3-year period ca. 1990. The lines are the fit of a four-degree polynomial model. All-time series start at the all-time minimum values recorded in August

The relations between domestic host availability, bug abundance, and blood-feeding behavior described above, in interaction with local host infection and infectiousness, determine household-level variations in the prevalence and abundance of domestic bugs infected with *T. cruzi*. The strong and positive association between household infection (especially children) and the presence or abundance of infected *T. infestans* or *P. megistus* has been documented across multiple settings (Mott et al.

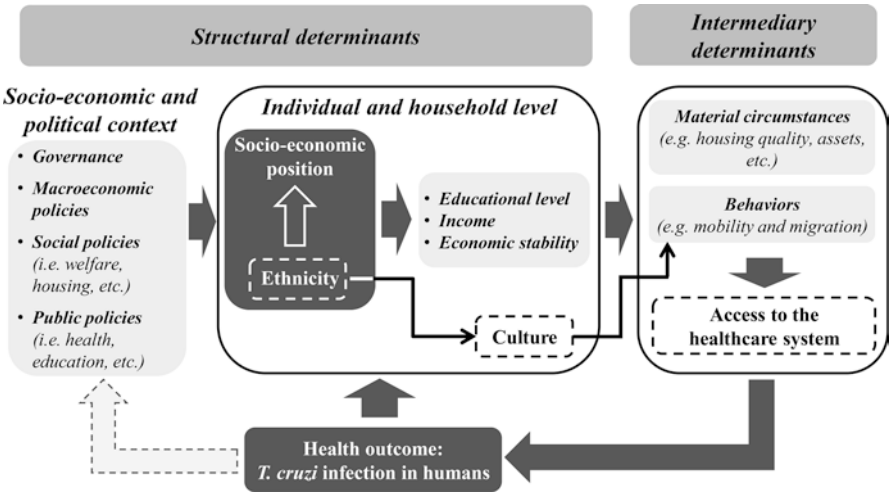

Fig. 5 Conceptual framework for the social determinants of health (SDHs) applied to Chagas disease. Adapted from Solar and Irwin (2010). The structural determinants are differentiated between those related to the socioeconomic and political context at national or regional levels and those pertaining to the individual and household level, as are the intermediary SDHs. The dashed light gray arrow indicates the possibility that negative health outcomes influence policy; this feedback is less likely to occur in the case of marginalized diseases

1976; Piesman et al. 1985; Gürtler et al. 1998a, 2005; Alroy et al. 2015; Cardinal et al. 2018; Fernández et al. 2019a). In a peri-urban community of Arequipa (Peru), child infection was instead associated with peridomestic infection in *T. infestans* (Levy et al. 2007). In rural villages of northwestern Argentina and northeast Brazil, human and vector infections were strongly and positively associated with the household presence and number of infected dogs (Cardinal et al. 2014; Gürtler et al. 1998a,b, 2005; Mott et al. 1978b).

In theory, the circulation of *T. cruzi* among multiple host species differing in reservoir competence might favor pathogen persistence (maintenance) or high pathogen abundance (amplification) or reduce both of them (the dilution effect) (Begon 2008). The empirical relations described above were embodied in a mathematical model of domestic transmission mediated by *T. infestans*, which accounted for host-species effects and seasonality (Cohen and Gürtler 2001). Having two or more *T. cruzi*-infected dogs disproportionately increased the prevalence of vector and human infection. Dogs acquired the infection within a few months of exposure, and 90–100% of dogs were infected by 3 years of age (Gürtler et al. 1996, 2007a). Although refractory to *T. cruzi* infection, the indoor occurrence of one or two nesting chickens boosted domestic bug populations beyond the constraints set by human and dog numbers and ultimately contributed to larger infected-bug abundances and human infection through frequent host shifts (Gürtler et al. 1998b) (see Sect. 2.5). Other model specifications reached similar conclusions: increasing the number of dogs would amplify the intensity of domestic transmission (Fabrizio et al. 2016;

Flores-Ferrer et al. 2019; Nouvellet et al. 2015; Peterson et al. 2015; Spagnuolo et al. 2012). The net effects of adding transmission non-competent hosts (chickens) on bug and host infection may vary with the precise details on the relative number and location of chickens, humans, and triatomine species involved and thus cannot be generalized to all ecological settings. The key finding is that chickens and selected domestic animals increase the equilibrium vector population size across triatomine species (e.g., Minter 1976; Dumonteil et al. 2018) and ultimately increase human-vector contact rates and exposure.

4 Social Determinants of Domestic Transmission

The social determinants of health (SDHs) condition both disease distribution and the ways they are handled (Manderson et al. 2009). Although the links between social factors and health outcomes have been widely recognized since the 1990s, the SDHs are often disregarded in biomedical research related to disease control because they fall outside the scope of traditional healthcare systems (Bizimana et al. 2015; Manderson et al. 2009). To address this issue, the World Health Organization has developed a conceptual framework to act upon the SDHs (Solar and Irwin 2010). This framework is based on the concept that "the social position of individuals and population groups is the main determinant of health inequalities within a community" by determining differential exposures to health-adverse conditions among individuals, differential consequences resulting from exposure (socioeconomic or health outcomes), and/or differential capabilities to recover (Solar and Irwin 2010).

The SDHs of Chagas disease include interrelated socioeconomic and demographic factors such as ethnicity, gender, occupation, educational level, and income, i.e., structural determinants (Ehrenberg and Ault 2005; Hotez et al. 2008; Aagaard-Hansen and Claire 2010; Solar and Irwin 2010). These determinants affect other factors more directly associated with disease exposure and outcome (i.e., intermediary determinants), such as household and dwelling characteristics.

4.1 Socioeconomic Factors

The social stratification of individuals and demographic groups results from, and also perpetuates, socioeconomic inequalities (Pluciński et al. 2013), leading some people to live in a state of relative or absolute poverty. Understanding poverty as a dynamic and multidimensional process (as opposed to a lack of resources) requires introducing the concept of social vulnerability, which considers the "defenselessness, insecurity, and exposure to risks, shocks and stress" experienced by households (Chambers 1989). The notion of social vulnerability summarizes the multiple, interrelated structural and intermediary determinants associated with the

socioeconomic position (SEP) of individuals and groups in a population. In the context of low- and middle-income countries, socioeconomic inequalities were represented using surrogate indicators such as educational attainment and household ownership of assets (Houweling et al. 2016). These indicators may only partially capture the full complexity of poverty. In particular, Chagas disease presents a disproportionately high disease burden on indigenous communities and poor rural peasants across Latin America (Hotez et al. 2008; Hotez 2014; Gürtler 2009). Although poverty has long been acknowledged as the main driver of Chagas disease risk (Ault 2007; Guhl et al. 2007; Briceño-León and Méndez Galván 2007; Gürtler 2009), evidence of the effects of socioeconomic inequalities is limited compared to other NTDs (Houweling et al. 2016; Fernández et al. 2019a).

The socioeconomic position of individuals and households is associated with other structural SDHs such as educational level, ethnicity, and income, which in turn will determine several intermediary SDHs associated with vector occurrence or invasion of human habitations, human exposure, and the resources required to deal with the consequences of vector exposure. However, finding appropriate measures of household SEP in low- and middle-income countries and in contexts of structural poverty is not trivial. Traditional income-based indices may fail to capture the full range of heterogeneity across household socioeconomic status (Booysen et al. 2008; Howe et al. 2012), particularly in communities where household monetary income is principally dependent on the scarce jobs available and on welfare support, as in many endemic areas for Chagas disease.

As an alternative, several studies have explored the effects of SEP-related intermediary and structural SDHs on house infestation. Most of them focused on housing quality and construction materials because of their association with refuge availability rather than as a measure of SEP (Levy et al. 2006; Gurevitz et al. 2011; Dumonteil et al. 2013; Bustamante et al. 2014; Gaspe et al. 2015). Other studies investigated the effects of other surrogates of SEP on house infestation with *T. infestans* and the occurrence of infected domestic vectors, sometimes using overcrowding and/or ownership of livestock (goat-equivalent index) as surrogate indices of household wealth (Gaspe et al. 2015; Cardinal et al. 2018). Overcrowding (i.e., human density in sleeping quarters) incorporates both household size and number of rooms and was closely and positively associated with domestic infestation and bug abundance (Gaspe et al. 2015). Overcrowding is expected to facilitate host finding and blood-feeding success on humans and most likely underlies the positive relation between the number of human occupants and domestic infestation with triatomine bugs (Marsden et al. 1982; Piesman et al. 1983; Levy et al. 2006; Campbell-Lendrum and Woodruff 2007; Provecho et al. 2017; Cardinal et al. 2018). The goat-equivalent index was weakly and inversely associated with house infestation with *T. infestans* and the occurrence of infected vectors in the Argentine Chaco (Gaspe et al. 2015; Cardinal et al. 2018); although it captured part of the variability between households and demographic groups, the effect was not statistically significant. Another study that included SEP as a risk factor for house infestation with *T. dimidiata* in Guatemala found a moderate association between house infestation and two summary indices representing household assets (cell phone and livestock)

and access to electricity (Bustamante et al. 2014). For intrusive species such as *T. dimidiata*, artificial light in the house or in nearby streets was positively associated with house infestation (Pacheco-Tucuch et al. 2012; Dumonteil et al. 2013).

Educational level has also been considered a surrogate of SEP in studies of house infestation in Yucatan (Dumonteil et al. 2013) and in the Argentine Chaco (Gaspe et al. 2015), where it showed a negative and significant association with domestic infestation. A direct causal pathway from increasing household educational levels to decreasing infestation may be related to access to information and receptivity to health education messages, which may translate into healthier practices (Solar and Irwin 2010). For example, education levels correlated directly with the severity of Chagas disease cardiomyopathy (Viotti et al. 2009). However, educational level (as determined by the duration of formal instruction) does not specify its quality nor health education through informal channels (Gaspe et al. 2015) and most likely reflects socioeconomic inequalities between households.

The association between human infection with *T. cruzi* and selected sociodemographic factors in rural endemic areas has been a recurrent focus of interest (e.g., Gürtler et al. 1998a, b, 2005; Levy et al. 2007; Samuels et al. 2013; Alroy et al. 2015; Cardinal et al. 2018). However, these studies did not address the combined effects of ecological and social variables due to limited data availability. While human infection increased with infected-bug abundance and the household presence or number of domestic dogs (see Sect. 3.5), a less consistent association was found with house construction quality (i.e., thatched roofs and cracks in the walls): some studies reported an inverse relation between infection and selected aspects of housing quality (Mott et al. 1978a; Gürtler et al. 1998a, b, 2005; Samuels et al. 2013), whereas others did not (Levy et al. 2007; Alroy et al. 2015).

The multiple SDHs related to poverty (e.g., poor-quality housing, household overcrowding, and low educational level) may be summarized into a social vulnerability index by means of multiple correspondence analysis (Fernández et al. 2019a). The concept of social vulnerability may be taken as an ex ante risk that a household will fall below the poverty line or, if already poor, will remain in poverty (Chaudhuri et al. 2002). When considered as a SDH, social vulnerability refers to a predisposition of certain individuals or groups to acquire the disease(s) in question, and their capacity to respond and access the healthcare system (Hagenlocher and Castro 2015; Bizimana et al. 2015). In creole and indigenous households in the Argentine Chaco, the social vulnerability index was positively associated with house infestation and the abundance of *T. cruzi*-infected domestic triatomines and was negatively correlated with the asset index and domestic insecticide use (Fernández et al. 2019a). This analysis clearly reveals the tight links between triatomine exposure and resource constraints, poor housing quality (suitable habitats), householders' prevention practices, and sociodemographic factors that reflect and perpetuate poverty. Most importantly, the social vulnerability index was positively associated with human infection after adjusting for other relevant demographic and ecological factors (Fernández et al. 2019b).

Access to health services, an important determinant related to SEP, also depends on health and infrastructure-related policies (Solar and Irwin 2010). Access to health services includes the availability of healthcare facilities and personnel and the distance and transportation means available to the household. In general, evidence of the association between socioeconomic status and access to health services is scattered and seems to be context-dependent (Raso et al. 2005; Fürst et al. 2009). Other barriers to healthcare access in endemic rural communities include alleged discriminatory behaviors within the health system, especially of indigenous and other vulnerable groups (Dell'Arciprete et al. 2014; Brierley et al. 2014). In the Argentine Chaco, domestic infestation was significantly lower in houses with greater access to health services, possibly reflecting the aggregation of non-infested, new houses built around healthcare posts or their improved access to insecticides or capacity to demand vector control actions (Fernández et al. 2019a). Access to healthcare and other services were one of the main reasons for household mobility within rural communities and relocation in the periphery of the local town (Sect. 4.3).

4.2 Ethnicity

The intersection between SEP and ethnicity can further increase the inequalities observed in Chagas disease endemic areas, as explained by the intersectional paradigm (Hankivsky and Christoffersen 2008). Multiple indigenous groups live in Chagas disease endemic areas (Hotez et al., 2008). In the Gran Chaco region, the seroprevalence of *T. cruzi* in indigenous peoples tended to exceed that of creole residents (Basombrio et al. 1999; Taranto et al. 2003; Biancardi et al. 2003; Diosque et al. 2004; Alonso et al. 2009; Sosa-Estani et al. 2009; Moretti et al. 2010; Lucero et al. 2016; Cardinal et al. 2018). House infestation rates with *T. infestans* were higher in indigenous households, consistent with their more precarious living conditions (Gurevitz et al. 2011; Gaspe et al. 2015, 2018; Provecho et al. 2017) and greater social vulnerability (Fernández et al. 2019a). Their dogs and cats also displayed greater infection prevalence than those owned by creoles (Cardinal et al. 2014). However, the statistical effects of ethnic background ceased to be significant when other ecological and socioeconomic variables more closely related to house infestation or domestic triatomine abundance were incorporated to these multimodel-based analyses (Gurevitz et al. 2011). The effects of ethnicity on human infection with *T. cruzi* may be more complex than anticipated: while a twofold greater risk occurred among indigenous people after adjusting for social vulnerability and other factors in a section of Pampa del Indio (Fernández et al. 2019b), no effects were observed in another rural section including a majority of creole households (Cardinal et al. 2018). Cultural factors associated with ethnic background (Arrom-Suhurt et al. 2018) likely affected human exposure to triatomines.

4.3 Human Migration and Mobility

Human migration and mobility can affect domestic transmission patterns by facilitating vector dispersal and modifying exposure to the vector and by introducing *T. cruzi*-infected people and nonhuman reservoir hosts. Migration from endemic regions to non-endemic countries (driven mostly by economic/labor reasons) expanded Chagas disease to Europe and non-endemic areas in North America, Japan, and Australia (Schmunis and Yadon 2010; Lee et al. 2013). There, vertical transmission became the main transmission route (Sicuri et al. 2011; Howard et al. 2014), followed by transmission via blood transfusion and organ transplantation before the implementation of prevention measures (Girolamo et al. 2011). The SEP of migrants in the receiving country and their access to health services created a new set of challenges for case detection and treatment (Ventura-Garcia et al. 2013). The same argument holds for migration from endemic rural areas to non-endemic areas within Latin America.

The steady rural-to-urban migration recorded during the twentieth century and projected for future decades, combined with increasing travel and transportation of goods from rural to peri-urban or urban areas, provides multiple routes of entry of triatomine bugs into habitats wrongly assumed not to be at risk of infestation. Consequently, several species of triatomines (including *T. infestans*, *T. dimidiata*, *Triatoma pallidipennis*, and *Mepraia spinolai*) colonized peri-urban and urban habitats and even invaded the top stories of city buildings through flight dispersal (Vallvé et al. 1996; Albarracin-Veizaga et al. 1999; Cattan et al. 2002; Ramsey et al. 2005; Levy et al. 2006; Guzman-Tapia et al. 2007; Medrano-Mercado et al. 2008; Lima et al. 2012; Gaspe et al. 2020). Peri-urban areas, defined as "the areas where the urban core intermingles with adjacent 'non-urban' systems" (MacGregor-Fors 2011), provide a transition between urban and rural areas. In Latin America, peri-urban areas frequently include precarious settlements where new migrants usually first settle, occupying vacant land with low land-tenure security (Levy et al. 2014). Therefore, migration and settlement patterns may represent relevant risk factors for house infestation and transmission of *T. cruzi* (Bayer et al. 2009; Delgado et al. 2013; Levy et al. 2014). The combination of substandard housing quality and proximity between houses facilitates triatomine invasion (Levy et al. 2006).

Rural communities in the Argentine Chaco displayed significant rates of rural-to-urban migration, internal mobility (i.e., moving within the study area: local movers), and return of migrants from urban areas (Fernández et al. 2019a). These migration and mobility patterns differed between ethnic groups: creoles displayed the traditional rural-to-urban movement (Briceño-León 2009), whereas indigenous (Qom) migration rates equaled internal mobility rates, with in-migration almost fully compensating out-migration. Qom mobility patterns are rooted in socioeconomic and cultural factors: nomadic traditions (Maidana 2011), formation of new families, household mobility to gain increased access to basic services (e.g., better water sources and school), and cultural reasons (decease of the head of family). Local mobility implied elevated house turnover rates causing substantial negative effects on extant house infestations (Gaspe et al. 2015, 2018) and possibly created

additional heterogeneities in human-vector contact rates (Stoddard et al. 2009). Local movers were likely exposed to lower, more variable infestations over time and greater chances of occupying an infested house than non-movers (Fernández et al., 2019a).

Human mobility in interaction with vector exposure created complex patterns in the seroprevalence rates of children from stable households (non-movers) and among movers (Fernández et al. 2019b). Among non-mover children, infection prevalence was sixfold greater when domestic premises were infested (as expected from prolonged vector exposure), whereas no significant association between child infection and domestic infestation occurred among movers (Fernández et al. 2019b). In a peri-urban area of Arequipa, domestic infestation was positively associated with land-tenure security (i.e., more established households, rather than more recent immigrants), suggesting that although land-tenure security may pave the way to improved house quality, it may also provide a more stable environment for triatomine establishment (Levy et al. 2014). Further research is needed to disentangle the effects of internal mobility on triatomine and human exposure dynamics and gauge the relative magnitude of these effects in endemic communities with different socioeconomic and demographic profiles.

4.4 Interactions Between Social and Ecological Factors

Understanding the domestic transmission of *T. cruzi* requires a better understanding of the interactions between ecological and social factors such as SEP, ethnicity, and household mobility. In rural communities of the Argentine Chaco, the intersection between ethnicity and SEP further combined with human mobility patterns: movers had higher social vulnerability than non-movers both within indigenous and creole households (Fernández et al. 2019a). The relative odds of human infection varied significantly with the interaction between social vulnerability index and infected-vector abundance: infected-vector abundance exerted lower effects (on a per capita basis) in households with higher social vulnerability (Fernández et al. 2019b). This unexpected effect may be explained by several nonexclusive reasons: (i) social vulnerability may also indicate past exposure to triatomines; (ii) households with higher social vulnerability had greater local mobility, suggesting putative vector exposures elsewhere; and (iii) more frequent human-triatomine contact given the greater overcrowding in households with higher social vulnerability.

5 Scaling Up from Household- to Population-Level Transmission

Although the domestic transmission of *T. cruzi* is clustered at a household level (Mott et al. 1976; Gürtler et al. 1998a, b; Levy et al. 2007; Cardinal et al. 2014,

2018; Fernández et al. 2019b), the combination of human mobility, spatial distribution of houses, and community-level variables (e.g., insecticide spraying or housing improvement efforts) may generate new properties at higher spatial scales. By scaling up from household-level dynamics to the population level, we may gain insight into the ecological and social factors associated with human infection and the impact of community-based vector control actions. The joint analysis of the spatial distribution of human and vector infection can shed light into the processes and factors associated with vector-borne transmission of *T. cruzi*. By integrating the spatial component of infection with household-level and individual-based risk factor analysis, we may identify transmission hotspots, create risk maps of *T. cruzi* infection, and stratify the affected areas for targeted control (Fortin and Dale 2005; Wiegand and Moloney 2014).

By contrast to vector-related research, the spatial component of human *T. cruzi* infection has rarely been investigated (Levy et al. 2007, 2009; Delgado et al. 2011; Fernández et al. 2019b). As stated by Houweling et al. (2016), "spatial clustering of infection because of geographic conditions, among other causes, is typical for most NTDs." Spatial clustering may also be context-specific and depend on the intersection between social and ecological factors. The spatial aggregation of house infestation (e.g., Cecere et al. 2004; Gaspe et al. 2015) and of human cases of *T. cruzi* infection across settings and scales indicates that spatial heterogeneity is the rule rather than the exception and that transmission is not restricted to the household. The underlying community-level processes possibly emerge from a combination of active and passive vector dispersal (Levy et al. 2006; Gurevitz et al. 2011: Gaspe et al. 2013, 2015; Provecho et al. 2017), the aggregation of more vulnerable households, and the effects of human mobility (Gaspe et al. 2015, 2018: Fernández et al. 2019a). These in turn can be affected by social networks and public policies. Integrating the ecological and social determinants of human infection with the spatial component is key to the design of more cost-effective vector control strategies in resource-constrained areas to identify higher-priority areas for targeted interventions oriented to suppress house (re)infestations, treat infected children, and thus reduce the current and future burden of disease.

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Chagas Disease Vector Control

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Abstract Most of the human infections with *Trypanosoma cruzi* are caused by triatomine species adapted to thrive in domestic habitats and blood-feed on humans. Residual house spraying with insecticides applied by public health personnel remains the mainstay of triatomine control, with pyrethroids being in use for nearly 40 years. Here, we review the vector control methods applied to triatomines of public health relevance. We examine the effectiveness and epidemiological impact of residual house spraying and housing improvement, and its main limitations, and focus on the reinfestation process and the putative sources of reinfestants. High levels of pyrethroid resistance associated with triatomine control failures appeared in the late 1990s and so far have remained restricted to northern Argentina and Bolivia. Peridomestic foci and effective vector surveillance at low bug densities remain the Achilles' heel of triatomine control programs. Other insecticide delivery systems of limited use include disposable fumigant canisters and insecticidal paints. Housing improvements and other multisectoral measures directed to prevent house colonization have not been exploited to its full potential and may play an important role for sustainable vector control across diseases. Integrating vector and disease management efforts may be the key to long-term program sustainability in the affected areas.

Keywords Housing improvement · Insecticide resistance · Vector control tactics · House reinfestation · Vector elimination · Epidemiological impact

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1 Background

The prominence of Chagas disease as a major neglected tropical disease in the Americas derives from its widespread occurrence affecting 7–10 million people with a chronic illness that causes variable degrees of incapacity and reduced lifetime and which imposes a massive economic burden on the affected individuals, families, and communities (Lee et al. 2013). Most human infections with *Trypanosoma cruzi* have been attributed to transmission mediated by triatomine bugs, especially by those species that establish and reproduce in domestic premises and while doing so blood-feed on humans. Since Carlos Chagas' discovery of vector-mediated transmission of *T. cruzi*, how to get rid of domestic triatomine bugs has garnered special attention.

Here we review the vector control methods applied to triatomines of recognized public health relevance. After summarizing the classifications of triatomine species and their significance, we describe the methods used for vector surveillance and their relative performance and limitations. Then we provide a historical overview of triatomine control methods, including main events such as the advent of synthetic insecticides, decentralization of vector control programs, and the creation of multinational initiatives for Chagas disease control. Because residual spraying with insecticides continues to dominate the scene of triatomine control since the mid-1940s, we describe its main effects on vector populations and parasite transmission, focusing on the process of reinfestation, its putative sources, and pyrethroid resistance. Bug population dynamics and transmission-related factors appear in a separate chapter of this book. Plant extracts, repellents, and other insecticides that have not reached Phase III trials and mathematical modeling of control intervention effects fall outside the scope of this chapter. A thorough account of triatomine population genetics, key to their dynamics, control, and transmission, can be found in Stevens and Dorn (2017).

We adopt some conventions of language. Following usual practice, the term "reinfested" applies to any site, house, or village sprayed with insecticides in which live triatomines are collected after the intervention had sufficient time to induce its effects, regardless of its preintervention status (i.e., infested or not). This definition reflects the fact that most vector control operations are unable to distinguish whether postintervention triatomines survived treatment (and therefore were not a truly new infestation) or in-migrated to the individual site, house, or village after treatment.

2 Species and Epidemiologic Relevance

Less than 20 species of Triatominae are involved in the transmission of *T. cruzi* infection to humans (Gourbière et al. 2012), and <10 triatomine species have adapted to thrive in domestic premises and feed regularly on humans and domestic animals (domestication; see chapter "Eco-Epidemiology of Vector-Borne

Transmission of *Trypanosoma Cruzi* in Domestic Habitats"). Noireau and Dujardin (2010) divided triatomine species in domestic, domiciliary, intrusive, and sylvatic based on their epidemiological relevance for transmission and control, as follows.

Triatoma infestans expresses the extreme of the evolutionary trend toward domesticity (Dujardin and Schofield 2004). It has widespread sylvatic foci in Bolivia and Chile (Rojas Cortez et al. 2007; Bacigalupo et al. 2010; Waleckx et al. 2012) and more discrete occurrences in the Argentine and Paraguayan Chaco (Ceballos et al. 2009, 2011; Rolón et al. 2011). This dual feature is also shared by *Rhodnius prolixus*, with extensive sylvatic foci in Venezuela and Colombia and no known sylvatic foci in Central America and southern Mexico prior to its elimination, and by *T. dimidiata* in Central America and Ecuador (Gorla and Noireau 2017). The intimate adaptation of *T. infestans* and *R. prolixus* to domestic habitats and significance as vectors of human infections, combined with other technical aspects, supported targeting them for regional elimination except where they have sylvatic foci (WHO 2002).

According to Noireau and Dujardin (2010), domiciliary species are able to establish viable colonies in human habitations (i.e., domestic premises) from their extensive sylvatic foci, but unlike domestic species, they would mainly disperse actively rather than passively through humans. The list includes *Triatoma dimidiata* and its various cryptic species in Central America, Ecuador, and northern Peru; *Triatoma brasiliensis* and closely related species or subspecies in northeastern Brazil; *Panstrongylus megistus* in central and eastern Brazil; *Triatoma barberi* and other members of the phyllosoma complex in Mexico; and *Triatoma maculata* and *Triatoma venosa* in Venezuela and Colombia, among others. Other species (e.g., *Rhodnius stali*, *Rhodnius pictipes*, *Panstrongylus lutzi*, *Panstrongylus rufotuberculatus*, and *Panstrongylus herreri*) frequently invaded or colonized domestic premises (Gorla and Noireau 2017). *Panstrongylus geniculatus* invaded domestic premises, fed on humans, and was implicated in human infections in Venezuela (Feliciangeli et al. 2004; Carrasco et al. 2005), as did *Triatoma tibiamaculata* in southeast Brazil, where it may have caused an oral outbreak of Chagas disease (Brenière et al. 2017). *Triatoma vitticeps* and other species may be in the process of adaptation to human dwellings.

Intrusive species invade domestic premises, contact humans to various degrees, but fail to establish domestic colonies (Noireau and Dujardin 2010). The main examples are *Rhodnius pallescens* in Panama and Colombia; *Rhodnius brethesi* in the Amazon basin (Coura and Junqueira 2015); *Rhodnius neglectus* in the Cerrado-Caatinga corridor in central Brazil; *Triatoma eratyrusiformis* in western Argentina (Cecere et al. 2016); and *Triatoma guasayana* and *Triatoma sordida* through most of the Gran Chaco ecoregion (Vazquez-Prokopec et al. 2008; Rodríguez-Planes et al. 2018). Yet *T. sordida* colonized domestic premises and was involved in human infections in Bolivia (Noireau et al. 1995). *Triatoma garciabesi* sustained abundant peridomestic populations but failed to invade and colonize domestic premises in Santiago del Estero over a 10-year period (Rodríguez-Planes et al. 2016), whereas it was frequently collected by householders in La Rioja (Cavallo et al. 2016). Six of 11 North American Triatominae with widespread sylvatic foci (*Triatoma gerstaeck-*

eri, *Triatoma lecticularia*, *Triatoma protracta*, *Triatoma recurva*, *Triatoma rubida*, and *Triatoma sanguisuga*) colonized domestic premises and nearby areas on some occasions and had high infection rates with *T. cruzi* averaging from 7.8 to 79.2%, and some caused life-threatening allergic reactions to their bites (Zeledón et al. 2012).

Sylvatic species appear to be virtually restricted to sylvatic habitats and encompass rare species such as *Cavernicola pilosa* and members of the Alberproseniini and Bolboderini tribes (Carcavallo et al. 1999; Dujardin and Schofield 2004).

Waleckx et al. (2015) raised attention to the fact that most of the 13 triatomine species they reviewed displayed a widely variable ability to invade and colonize human dwellings, even within the same species, and thus were hard to fit in classification schemes that lack a quantitative base. Abad-Franch (2016) emphasized that all triatomine species have sylvatic foci at least in a section of their range, including the "domestic" species, and devised a four-level hierarchical system for strictly operational purposes.

Semantic issues are important for a correct formulation of the problem and prescribing appropriate control actions. In the Chagas disease literature, domestic is frequently taken as equivalent to domiciliary (or intradomiciliary) and refers to human sleeping accommodations and to the site of bug occurrence or collection. This is how we use "domestic" in this chapter. The distinction between domestic and peridomestic, or between peridomestic and sylvatic, is often unclear because the domicile is sometimes equaled to the housing unit, which frequently includes peridomestic annexes. This may lead to consider strictly sylvatic or peridomestic species such as *T. guasayana* or *T. garciabesi* as "domiciliary" despite of their inability to colonize domestic premises and of its marginal or nil infection with *T. cruzi* in the Gran Chaco. In some rural contexts with undefined property lines, the diffuse limits between peridomestic and sylvatic habitats create a new space for interaction between free-ranging domestic animals (goats, dogs) or wild rodents and various species such as *T. guasayana* or *T. eratyrusiformis* (Vazquez-Prokopec et al. 2008; Cecere et al. 2016). The exact definition of the peridomestic space is elusive (Walter et al. 2007) and may include permanent (animal enclosures) or temporary structures (such as brick piles and tiles) and natural vegetation (palm trees). Sylvatic habitats are not considered targets for control actions.

The rich diversity of ecological patterns described above defies any simple classification system. Any domestic colony or triatomine found in human sleeping accommodations is a potential threat or nuisance and therefore should be removed regardless of other details. The how, when, and who will cope with these situations is less clear and should be dealt with on a case-by-case basis.

3 Vector Detection Methods

Vector surveillance methods are an essential component of a decision support system. They are required to establish whether a house or village should be treated with insecticide and to monitor its effects, to provide reliable estimates of village- or

region-wide house infestation indices for operational decisions at a larger scale, and to declare whether the target vector has been eliminated from a district or region. However, detecting the presence of triatomines in houses is difficult because they hide in cracks, thatched roofs, furniture, clothes, and other goods during the daytime. A key point is that the detectability of house infestations decreases with decreasing local bug abundance regardless of the species, setting, and sampling method used. None of the extant methods provides a "gold standard" to establish whether a house is infested or not.

Active Methods The traditional method used to assess the occurrence and intensity of house infestation with triatomines has been timed manual collections (TMC). Using this method, skilled vector control personnel aided with forceps and a flashlight search and capture triatomines (including exuviae, eggshells, or dead bugs) during a fixed search time per house, frequently assisted with a pyrethroid-based aerosol to dislodge the insects from their refuges (Schofield 1978; Pinchin et al. 1981b). Bug collections are usually stratified by domestic and peridomestic habitats. The results are expressed qualitatively as an index of house infestation (presence of at least one live triatomine barring eggs) or colonization (presence of live nymphs) or quantitatively as the number of live triatomines caught per unit of search effort (per person-hour or fraction). The latter metric is a proportional correlate of absolute bug abundance (Rabinovich et al. 1995).

The outcome of TMC is crucially dependent on the number and skill of bug collectors and the length of time spent searching each house (Schofield 1978). It is also affected by the physical structure of triatomine habitats; temperature during bug searches (most triatomines may hardly move below ~15 °C); bug size, which affects the chances of sighting and catching the insects (Rabinovich et al. 1995); and local bug abundance. The sensitivity of TMC using a dislodging agent was 70–77% in mud-and-thatch houses infested with *T. infestans* in northwest Argentina (Gürtler et al. 1995) and 28.3% for three triatomine species combined in northeast Brazil (Abad-Franch et al. 2014). Therefore, the outcomes of vector control trials measured by TMC *only* will underestimate the true prevalence of infestation by a widely variable factor. Using additional bug sampling methods (e.g., insecticide knockdown, sensing devices, or traps) may provide an adequate solution.

House-dwellers may also be stimulated to collect triatomines, serving as an additional method for long-term monitoring of the low-density infestations that occur after control actions or of intrusive triatomine species (Gürtler et al. 1999; Dumonteil et al. 2009; Abad-Franch et al. 2011; Gaspe et al. 2018). However, householders' detectability of peridomestic foci was less satisfactory, perhaps because they did not perceive them as a potential hazard or nuisance (Cecere et al. 2019) and failed to detect early triatomine stages (Cavallo et al. 2018). Participatory approaches scaled up to the state level gathered valuable information on the geographic and seasonal distribution of triatomines and their infection with *T. cruzi* in Texas (Curtis-Robles et al. 2015). If adequately instructed and stimulated, schoolchildren can be very effective in collecting bugs during the vector surveillance phase (Crocco et al. 2005).

Householders' reports of bug infestations to a designated receptor or healthcare post without returning specimens may be used as a cursory index of house infestation and for screening purposes. Further verification with an independent method is needed before implementing control actions. Enhancing motivation and participation are key to all avenues of community-based vector surveillance.

Passive Methods Passive methods generally involve the use of shelter boxes fixed to walls for prolonged periods to increase the chance of detecting indirect signs of triatomines (fecal smears, eggs, or exuviae) or live or dead insects in its interior. Sensing devices for indoor use include several designs of cardboard boxes (e.g., Gómez-Núñez 1965; Wisnivesky-Colli et al. 1988) with an internal structure that provides adequate refuge and exploits some triatomine behavior features (i.e., negative phototaxis, thigmotaxis, and preference for dry sites). Sheets of typing paper (García-Zapata and Marsden 1993) or calendars (Rojas de Arias et al. 1999) have been used for a similar purpose. For outdoor use, the devices have to withstand exposure to sun or rain and provide good refuge: examples include bamboo canes (García-Zapata and Marsden 1993) and recycled tetra brik boxes (Vazquez-Prokopec et al. 2002). Comparisons between sensing devices and TMC using a dislodging aerosol yielded variable outcomes, probably owing to large variations in setting up procedures and assessment criteria.

Traps Sticky traps using a live bait (i.e., Noireau traps) were successfully used to collect sylvatic triatomines in hardly accessible habitats such as palm tree crowns or burrows (Noireau et al. 1999; Ceballos et al. 2009; Brenière et al. 2013). Bug-sensing devices combining the use of a shelter box, synthetic host odorants (e.g., hexanal, nonanal, octanal, benzaldehyde), and glue were effective in detecting domestic and peridomestic infestations with *T. infestans* and the invasion of *T. sordida* in two field trials (Rojas de Arias et al. 2012). In a matched comparison trial, a double-sided sticky trap (Rojas-Cortez 2007) revealed several low-density infestations with *T. infestans* that had been missed by TMC using a dislodging agent (Enriquez et al. 2020). The sticky trap apparently intercepted and trapped vectors seeking a blood meal host and may be especially apt for community-based triatomine surveillance and verification of the current status of house infestation in region- or district-wide elimination programs that apply for certification.

4 Historical Overview of Triatomine Control

The discovery that the organochlorine DDT was effective against multiple insect pests and disease vectors heralded the era of chemical control from the 1940s onward. Before that, there was no effective and practical means to suppress house infestations with triatomines. Initial bug control attempts included the fumigation of cyanide or methyl bromide and use of flamethrowers (Dias and Schofield 2004). DDT was not as effective against triatomine bugs as it was for other insect vectors and was replaced by another organochlorine insecticide, benzene-hexachloride

(BHC), whose gamma isomer (lindane or gammexane) is the only active ingredient against triatomines. Experimental evidence showed that all triatomine stages were susceptible to BHC; the larger the body mass (i.e., stage), the larger the lethal insecticide dose required, and duration of starvation affected the bugs' susceptibility to the insecticide (Zerba 1999). Other important requirements for large-scale triatomine control or elimination were that all stages of the main vector species were within the house compound and the fact that triatomine population growth rates were relatively lower than for other insect vectors.

Following experimental evidence (Busvine and Barnes 1947), the first field trials were conducted in Brazil and Argentina (Dias and Pellegrino 1948; Romaña and Abalos 1948). They showed BHC was able to suppress domestic triatomines at 0.5–2 g of active ingredient (a.i.) per m^2 of sprayed surface in two spray cycles 1–6 m apart, designed to kill any residual eggs or nymphs (Gualtieri et al. 1985; WHO 1991). Dieldrin, another organochlorine more toxic to humans and less effective than BHC, was used in Venezuela (1947–1971) until *R. prolixus* populations developed resistance against both dieldrin and BHC.

Using the vertical organizational structure of yellow fever and malaria control programs, large-scale insecticide spraying campaigns were launched in Argentina and Venezuela. These campaigns subsequently led to the official establishment of national Chagas control programs in 1963 (Segura 2002) and 1966 (Aché and Matos 2001), respectively. The long-held notion of eradication was quite influential at that time both in Venezuela (following success in malaria control) and Argentina (where *Aedes aegypti* had been eliminated). Region-wide triatomine control programs were also established in other countries. In Brazil, following state-based control efforts in Minas Gerais by the mid-1950s, the national control program was reformulated in 1975, and a nationwide insecticide spraying campaign was initiated in 1983 (Dias 2002). These large-scale operations reduced house infestations and transmission and laid the foundations for subsequent progress during the 1990s (see Sect. 5.1.1).

Carbamates (propoxur since 1968 and bendiocarb, Table 1) were effective against triatomines though more expensive and more toxic to vertebrates than BHC at the minimum dose required (Gualtieri et al. 1985). Bendiocarb applied at 500 mg/m^2 reduced house infestation rates with *P. megistus* from 18% to 7% within 6–12 months post-application (Sherlock et al. 1983). Unlike their predecessors, organophosphorus insecticides (malathion and fenitrothion, gradually introduced since 1975) were more effective at lower doses and killed triatomine eggs, suggesting bug elimination would require fewer annual sprays (Martínez et al. 1975). Although organophosphorus insecticides were much less environmentally aggressive than BHC, their moderate toxicity to humans and long-lasting unpleasant smell frequently led villagers to reject house spraying operations.

The advent of pyrethroid insecticides by the mid-1970s, including deltamethrin, permethrin, and cypermethrin, started a new era of promise of greater effectiveness at lower doses, faster degradation of insecticide molecules, and lower mammalian toxicity than those displayed by its predecessors (Lhoste 1982), albeit at a greater cost per house unit treated. Preliminary small-scale field trials demonstrated the efficacy of deltamethrin and other pyrethroids against triatomine bugs (Colas and

Table 1 Formulations and field concentrations of insecticides commonly used for spraying against triatomines according to WHO (1991, 2002)

Insecticide	Formulation	Field concentration (g a.i./m^2)
Organochlorines		
BHC	–	0.5
Dieldrin	WP	1–2
Carbamates		
Propoxur	EC	1
Bendiocarb	SC	0.4–1
Organophosphate		
Malathion	EC	2
Fenithrotion	EC	1
Pyrethroids		
Deltamethrin	SC, EC	0.025–0.05
Permethrin	WP	0.1–2
Etofenprox	WP	0.125–0.25
Bifenthrin	WP	0.05
Cyfluthrin	WP	0.045–0.05
λ-Cyhalothrin	WP	0.03
Cypermethrin	WP, EC	0.125
β-Cyfluthrin	SC	0.025
β-Cypermethrin	SC	0.05
α-Cypermethrin	SC, WP	0.025–0.05

BHC benzene-hexachloride, *WP* wettable powder, *EC* emulsifiable concentrate, *SC* suspension concentrate, *g a.i./m*^{2} grams of active ingredient per square meter

Delabarre 1982; Pinchin et al. 1980, 1981a; Gualtieri et al. 1984). Pyrethroids soon replaced other insecticides over the late 1980s and 1990s.

The major economic and political changes that swept across Latin America over the 1980s combined with the decentralization of health services transformed the vertical structure of Chagas disease vector control programs and weakened their operational capacity (Yadón et al. 2006). Paradoxically, this occurred while the large, hidden magnitude of Chagas disease started to emerge (WHO 1991, 2002). Two responses to this untoward scenario were to strengthen the links between triatomine control programs and primary healthcare systems, especially during the surveillance (vigilance) phase (e.g., Marsden 1984; Chuit et al. 1992), and to promote community-based insecticide spraying operations conducted by householders themselves (Segura 2002). Both responses can be traced back to the influences of the Alma-Ata convention held in 1978.

Third-generation pyrethroids (i.e., cyano-pyrethroids, which contain the most active isomers) have been used against triatomines since the 1990s (Table 1) (Rozendaal 1997; Schofield 2000, 2001). Technical-grade lambda-cyhalothrin and alpha-cypermethrin were substantially more effective than cyfluthrin or deltamethrin on the major vector species (Zerba 1999; Oliveira Filho 1999). Suspension concentrate (SC) and wettable powder (WP) formulations provided the best profile

of toxicological and practical advantages and thus remained as the first options for triatomine control programs (Fig. 1). Emulsifiable concentrate (EC) formulations did not provide sufficient residual activity on porous surfaces because they have smaller insecticide particles that quickly disappear from the treated surface. Modern microencapsulated formulations of insecticides protect the active ingredients from rapid degradation in outdoor habitats and thus appear as a cost-effective option for peridomestic triatomine populations (e.g., Gorla et al. 2015).

Chagas disease control gained momentum over the 1990s through the creation of intergovernmental initiatives to interrupt both vector-borne and transfusional transmission of *T. cruzi* at regional scales (WHO 2002): the Southern Cone (1991); the Andean Pact (1997); Central America (1997), later joined by Mexico; and the Amazon basin initiative (2004). All of them were created under the aegis of the Pan American Health Organization. At the outset, the status of transmission and disease control was extremely heterogeneous across Latin America: while Brazil, Chile, and Uruguay were in an advanced state of control, several of the affected countries lacked properly structured triatomine control programs. The multinational initiatives assisted them in establishing new programs, strengthened the capacities of

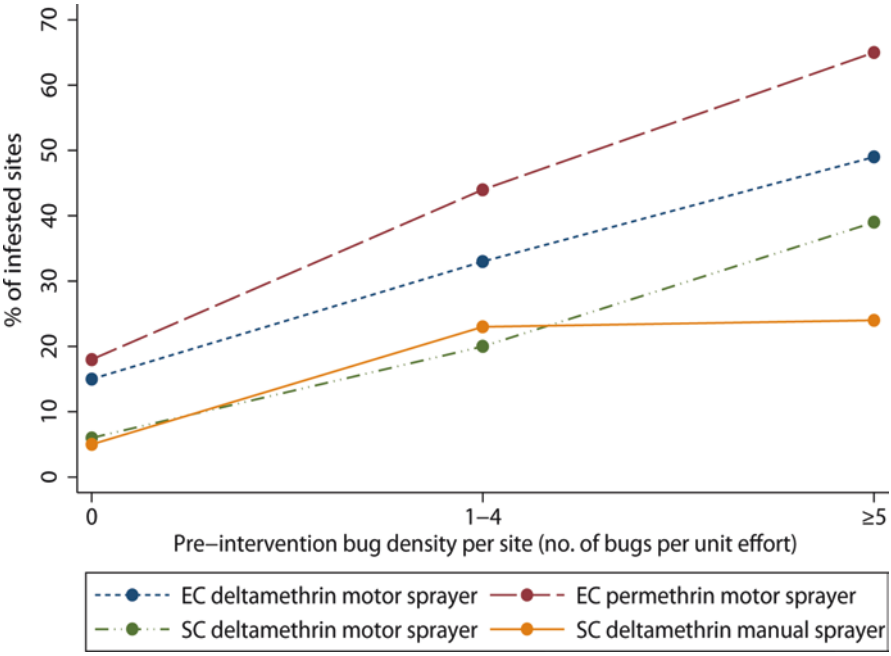

Fig. 1 Prevalence of site-specific infestation with *T. infestans* 1 year posttreatment according to preintervention local bug abundance in peridomestic habitats treated with suspension concentrate (SC) deltamethrin applied with manual compression or motor sprayers and emulsion concentrate (EC) deltamethrin or EC permethrin applied with motor sprayers, Olta, 1999–2000. (Redrawn from Gürtler et al. 2004)

existing programs, and created an international forum to periodically review the degree of progress.

The regional programs targeted some species for regional elimination (e.g., *T. infestans*, except in Bolivia) and others for control (e.g., *T. dimidiata* in Central America). The official formalization of the Southern Cone Initiative by late 1991 set among its goals for the year 2000 "to eliminate *T. infestans* of dwellings and peridomestic ecotopes of endemic and probably endemic areas" (Schofield and Dias 1999). Although the goals and targets were clear, the notions on how reinfestation would proceed and eventually threaten success were ill founded (see section Sources of Reinfestation).

These renewed control efforts made great strides toward achieving the set goals. Following the initial successes in the Southern Cone (Silveira et al. 2002), the long-term labor of the initiatives documented nationwide success in 11 of the 20 recognized endemic countries and within defined sections of other countries and more lately addressed the challenges of congenital transmission and etiologic treatment (Pan American Health Organization 2018). Most of its underpinnings and outcomes have been widely reviewed (Silveira et al. 2002; Dias and Schofield 2004; Yamagata and Nakagawa 2006, Gorla and Hashimoto 2017; Peterson et al. 2019).

The emergence of pyrethroid resistance in a well-defined region of the Southern Cone by the late 1990s stimulated the search for other candidate insecticides and control methods. For example, fipronil (a phenylpyrazole broad-spectrum insecticide) proved effective against *T. infestans* and *R. neglectus* during 3 months when applied to lime-coated or uncoated mud blocks at 100–200 mg a.i./m^2 (Rojas de Arias and Fournet 2002) and was effective against pyrethroid-resistant populations from Argentina but not from Bolivia.

5 Vector Control Methods

Triatomine control is mainly achieved through residual insecticide spraying of walls, roof, and peridomestic outhouses and reduction of vector resting places in human habitations and peridomestic structures (Rozendaal 1997; WHO 2017).

Community Participation and Health Education Both components play a key role in sustainable vector control regardless of the method applied and have been considered prerequisites to launching any intervention since the 1990s. Community workshops and other media dissemination means may be used to inform householders of disease basic aspects, control actions, diagnosis, and treatment. Yet school- or media-based health education interventions notably lag far behind vector control efforts.

Community participation entails a large spectrum of involvement in disease control (Rifkin 2009). At the very least, householders and local leaders must be informed of local disease status and planned vector control actions and requested to grant permission to access their premises and prepare them for spraying. Householders

may also participate by reporting or providing proof of bug infestation to a local healthcare post and thus adopt an active role during the vigilance phase (Marsden 1984; Abad-Franch et al. 2011). At the other extreme of the spectrum, householders may assist in the design and implementation of locally adapted vector control actions, such as plastering of walls and modification of peridomestic structures (Monroy et al. 2009), and treat their homes with insecticides (Sherlock and Piesman 1984; Segura 2002; Cecere et al. 2019). Community participation in the design, planning, and evaluation of interventions targeting triatomines remains virtually absent (Abad-Franch et al. 2011). Appropriate stimulus, training, and resources are essential to sustain community engagement in vector control, especially when triatomine densities and risk of infection are low.

Householders influence the process of house infestation and reinfestation through their attitudes and practices in specific social and economic contexts (see chapter "Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma Cruzi* in Domestic Habitats"). For example, household mobility and associated relocation may impact negatively on extant triatomine populations through habitat destruction and host emigration and increase vector dispersal (both active and passive); household relocation may also create new suitable habitats free from insecticide treatment (Gaspe et al. 2018). House-dwellers may plaster house walls; screen windows; tear down infested chicken coops and build new ones; and apply smoke to repel triatomines or nonproprietary insecticides.

Householder insecticide use in domestic premises was frequently inversely related to domestic bug abundance (e.g., Gaspe et al. 2015). Householders' control actions weaken the links between peridomestic and domestic triatomine foci and decouple their dynamics (i.e., break the positive association between peridomestic and domestic infestation with *T. infestans*, e.g., Cecere et al. 2019). If control or prevention efforts are systematic enough, they may eventually create a source-sink dynamics between peridomestic and domestic habitats, respectively. This mechanism, combined with recurrent insecticide spraying campaigns and gradual improvement of rural housing, may explain some field patterns (Gorla and Hashimoto 2017, p 239).

5.1 Chemical Vector Control

Residual house spraying with insecticides applied by public health personnel has been the mainstay of triatomine control and continues to be the standard reference for method comparison trials (Rozendaal 1997; WHO 2002; Gorla and Hashimoto 2017). Other insecticide delivery systems of more limited use involve disposable fumigant canisters and insecticidal paints. Experimental trials of insecticide-impregnated bednets, dog collars, and materials and spot-on or pour-on insecticides have shown promising results.

Residual House Spraying

House treatment traditionally involved the application of insecticides to inner and outer walls, interior ceilings, beds, animal shelters, and storage areas using manual compression sprayers. Clothes, bedding, and storage boxes were usually taken outside and left under the sunlight. The term "indoor residual spraying" (e.g., WHO 2012), borrowed from mosquito control programs, is misleading when applied to the main triatomine species, which frequently infest peridomestic structures and for which standard good practice dictates spraying with insecticides both domestic and peridomestic structures.

Pyrethroid Effects Pyrethroids increase the locomotory activity (hyperactivity) of insects and then cause incoordination, paralysis (leading to the knockdown effect), prostration (with eventual recovery), and death, depending on insect body mass, dose, and duration of exposure (Lhoste 1982). These effects have been verified in triatomines (Alzogaray and Zerba 2001a). Moreover, pyrethroids exert both spatial repellent and contact irritant effects in mosquitoes (e.g., Grieco et al. 2007). In Triatominae, some pyrethroids lacked repellent effects in laboratory assays (Alzogaray and Zerba 2001b), whereas they caused increased irritation, mobility, and reduced blood intake in other experimental setups (Kroeger et al. 1999; Diotaiuti et al. 2000; Reithinger et al. 2005; Maloney et al. 2013).

The residual efficacy of pyrethroids over time post-application depends on the substrate on which it is applied, type of formulation, target dose and application procedures, triatomine species and stage, and other environmental conditions. Temperature effects are especially relevant because pyrethroids' toxic effects on insects decrease with increasing temperature (Lhoste 1982; Alzogaray and Zerba 1993). As temperature affects insect mobility, exposure to treated surfaces, and pyrethroid toxicity, the effectiveness of insecticide spraying is expected to vary seasonally (c.f., Gorla 1991).

In field bioassays, pyrethroids applied indoors retained some residual effects on *T. infestans* over widely variable (3–12 months) periods after application (Table 2). The wide range in estimates is likely related to variations in substrate, triatomine instar used in bioassays, duration of exposure, and post-exposure observation times. Porous surfaces, such as mud walls, sequester insecticide molecules, whereas wooden poles or walls allow extended efficacy (Rojas de Arias et al. 2003, 2004). Insecticide molecules persist longer on painted walls or wood and in lime-coated mud. In outdoor peridomestic sites, however, sunlight, rainfall, and dust degrade pyrethroid molecules and reduce their residual activity to a few days (Gürtler et al. 2004). Because triatomine eggs may take 2–3 weeks to hatch depending on ambient temperature, in outdoor habitats first-instar nymphs may emerge after residual effects disappear.

Intervention Trials with Pyrethroids The initial small-scale field trials of pyrethroids against triatomines created a false sense of immediate and prolonged success. For example, "It has been demonstrated that these products keep houses and

Table 2 Evaluations of the residual activity of pyrethroid insecticides used for triatomine control

Observation time after exposure (h)					
Insecticide (formulation, dose)	Triatomine species	Stage	(Habitat, location)	Mortality (%) over time post-treatment	References
Deltamethrin (5% SC, 25 mg/m^2)	*T. infestans*	Second	72 (domicile walls, Brazil)	6%, 7 m	Diotaiuti and Pinto (1991)
	T. infestans	Third	72 (domicile walls, Brazil)	33%, 3 m; 53%, 13%, 9 m	Diotaiuti and Pinto (1991)
	T. infestans	Fifth	72 (domicile walls, Perú)*	95%, 1 day; 88%, 1 m; 60%, 3 m; 34%, 4 m	Palomino et al. (2007)
	T. sordida	Second	72 (domicile walls, Brazil)	74%, 7 m	Diotaiuti and Pinto (1991)
	T. sordida	Third	72 (domicile walls, Brazil)	16%, 3 m; 35%, 7 m; 32%, 9 m	Diotaiuti and Pinto (1991)
Deltamethrin (2.5% SC,?)	*T. infestans*	Fifth	8 (domicile, Argentina)	3 m,?	Gualtieri et al. (1984)
Deltamethrin (2.5% SC, 25 mg/m^2)	*T. infestans*	Fifth	24 (domicile walls, Bolivia)	~90%, 1 m; ~30%, 3 m; 0%, 6 m; ~5%, 9 m; ~30%, 12 m	Guillen et al. (1997) +
	T. infestans	Third	24 (domicile, Paraguay)*	57–100%, 1 m; 0–100%, 3 m; 0–53%, 6 m	Rojas de Arias et al. (2003)
	T. infestans	Fifth	24 (peridomicile, Argentina)*	0–100%, 7 day	Gürtler et al. (2004) ++
Deltamethrin (5% WP, 25 mg/m^2)	*T. infestans*	Third	72 (domicile walls, Brazil)	23%, 3 m; 40%, 7 m; 14%, 9 m	Diotaiuti and Pinto (1991)
	T. sordida	Third	72 (domicile walls, Brazil)	9%, 3 m; 37%, 7 m; 27%, 9 m	Diotaiuti and Pinto (1991)
Deltamethrin (2.5% WP, 25 mg/m^2)	*T. infestans*	Third	24 (domicile, Paraguay)*	43–83%, 1 m; 0–27%, 3 m; 7–13%, 6 m	Rojas de Arias et al. (2003)
Deltamethrin (1.5% EC, 25 mg/m^2)	*T. infestans*	Fifth	24 (peridomicile, Argentina)*	20–37%, 7 day	Gürtler et al. (2004) ++
Deltamethrin (1% EC)	*T. infestans*	Fifth	8 (domicile, Argentina)	1 m,?	Gualtieri et al. (1984)
Cyfluthrin (12.5% SC, 25 mg/m^2)	*T. infestans*	Third	24 (domicile, Paraguay)*	0–77%, 1 m; 13–60%, 3 m; 0–100%, 6 m	Rojas de Arias et al. (2003)

(continued)

Table 2 (continued)

Observation time after exposure (h)					
Insecticide (formulation, dose)	Triatomine species	Stage	(Habitat, location)	Mortality (%) over time post-treatment	References
Cis-permethrin (10% EC, 170 mg/m^2)	*T. infestans*	Fifth	24 (peridomicile, Argentina)*	27–30%, 7 day; 20–27%, 11 day	Gürtler et al. (2004) ++
λ-Cyhalothrin (2.5% SC, 1.87 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm- leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m; 97%, 6 m; ~80%, 9 m; ~60%, 12 m; ~40%, 15 m; 33%, 18 m	Mazariego-Arana et al. (2002)
λ-Cyhalothrin (2.5% SC, 3.75 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm- leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m, 9 m; 97%, 12 m; ~75%, 15 m; 63%, 18 m	Mazariego-Arana et al. (2002)
λ-Cyhalothrin (2.5% SC, 5.63 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm- leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m, 9 m; 96%, 12 m; 93%, 15 m; 87%, 18 m	Mazariego-Arana et al. (2002)
λ-Cyhalothrin (2.5% SC, 12.5 mg/m^2)	*R. prolixus*	Third	1 (impregnated fabrics)++	100%, 3 m	Kroeger et al. (2003)
λ-Cyhalothrin (10% WP, 30 mg/m^2)	*T. infestans*	Second	72 (domicile walls)	55%, 12 m	Diotaiuti and Pinto (1991)
	T. infestans	Third	72 (domicile walls)	2%, 3 m; 53% 7 m; 21%, 9 m	Diotaiuti and Pinto (1991)
	T. sordida	Second	72 (domicile walls)	83%, 12 m	Diotaiuti and Pinto (1991)
	T. sordida	Third	72 (domicile walls)	27%, 3 m; 30%, 7 m; 23%, 9 m	Diotaiuti and Pinto (1991)
λ-Cyhalothrin (10% WP, 109 mg/m^2)	*T. infestans*	First	72 (domicile walls, Brazil)*	0–50%, 12 m	Ferro et al. (1995)
	T. infestans	Fifth	72 (domicile walls, Brazil)*	0–60%, 1 m; 0–10%, 6 m; 0–10%, 12 m	Ferro et al. (1995)
λ-Cyhalothrin (10% WP, 8.6 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm-leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m, 6 m; 93%, 9 m; ~85%, 12 m; ~70%, 15 m; ~55%, 18 m	Mazariego-Arana et al. (2002)

(continued)

Table 2 (continued)

Observation time after exposure (h)					
Insecticide (formulation, dose)	Triatomine species	Stage	(Habitat, location)	Mortality (%) over time post-treatment	References
λ-Cyhalothrin (10% WP, 16.7 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm-leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m, 6 m, 9 m, 12 m; 93%, 15 m; ~90%, 18 m	Mazariego-Arana et al. (2002)
λ-Cyhalothrin (10% WP, 23.7 mg/m^2)	*R. prolixus*	Fifth	24 (domicile palm- leaved roofs, Mexico)	100%, 1 day, 1 m, 3 m, 6 m, 9 m, 12 m, 15 m; 97%, 18 m	Mazariego-Arana et al. (2002)
λ-Cyhalothrin (10% WP, 30 mg/m^2)	*T. infestans*	Third	24 (domicile, Paraguay)*	33–100%, 1 m; 0–100%, 3 m; 0–46%, 6 m	Rojas de Arias et al. (2003)

SC suspension concentrate (flowable concentrate), *EC*, emulsifiable concentrate, *WP* wettable powder, *m* months after treatment with insecticidal effects

*Insecticides applied in different substrates (i.e., wooden posts, wattle with mud or mud painted with lime, cement/mud/wood/lime-coated/soil-cement blocks)

+Bug mortality at 14 day after a 1 day exposure

++Bug mortality at 75 day post-exposure after 7 days post-spraying

++Bug mortality at 12 h post-exposure

peridomestic structures free from vectors for about 2 years (WHO 1991, p. 48).", but see Colas and Delabarre (1982, p 269). The following review of the efficacy of large-scale intervention trials, in which bug infestations were monitored after residual insecticide spraying mostly by TMC, displays more complex, heterogeneous patterns.

For *T. infestans*, two main patterns emerge: trials that completely suppressed domestic infestations over 6–12 months post-application (e.g., Guillén et al. 1997; Zerba et al. 1997; Rojas de Arias et al. 2004) and trials that substantially reduced bug abundance but in which a variable fraction of domestic premises were infested by 3 months post-application regardless of the pyrethroid applied (e.g., Marcondes 1989; Oliveira Filho 1989; Schofield 1994, p. 62). Randomized trials conducted in semi-arid rural areas of the Argentine Chaco demonstrated that the effectiveness of various pyrethroid formulations applied with different spray gear was not able to suppress the *peridomestic populations* of *T. infestans* (Gürtler et al. 2004; Cecere et al. 2006a, 2013). Better results were obtained in peridomestic structures when SC deltamethrin or SC beta-cypermethrin was applied using twice the standard target dose (Cecere et al. 2006a, 2013; Vazquez-Prokopec 2007). Using motor sprayers did not improve the efficacy of residual insecticide spraying (Cecere et al. 2006a; Carbajal de la Fuente et al. 2017).

For *R. prolixus* in Guatemala, where it lacked sylvatic foci, application of SC deltamethrin or WP beta-cyfluthrin (both at 25 mg/m^2) virtually suppressed infestations by 6–9 months post-application, whereas the effects on *T. dimidiata* were less

satisfactory and heterogeneous between villages (Nakagawa et al. 2003), likewise in other triatomine species with extensive sylvatic foci as described below. For *R. prolixus* in Barinas (Venezuela), a large insecticide trial including 526 houses sprayed either with fenitrothion (2 g/m^2) or deltamethrin (25 mg/m^2) revealed large domestic and peridomestic infestation rates 1 year post-application, ranging from 32 and 17% (fenitrothion) to 19 and 11% (deltamethrin), respectively (Sánchez-Martin et al. 2006).

For *T. dimidiata* in Yucatan (Mexico), house reinfestation with adult bugs was detected as early as 4 months post-application and most likely originated from intrusive sylvatic triatomines (Dumonteil et al. 2004). In Guatemala, a single application of WP deltamethrin at standard dose reduced domestic infestation or colonization and bug infection rates with *T. cruzi*, whereas peridomestic infestations were virtually unaffected (Monroy et al. 2009). In contrast, application of WP cyfluthrin virtually eliminated all infestations with *T. dimidiata* in Nicaragua regardless of treatment strategy (Acevedo et al. 2000).

For *Triatoma barberi* and *Triatoma pallidipennis* in Mexico, application of WP cyfluthrin caused greater impacts on domestic rather than peridomestic infestations and outperformed SC deltamethrin or WP bifenthrin (Ramsey et al. 2003). For *Rhodnius ecuadoriensis* in Ecuador, houses with infested peridomestic sites selectively treated with deltamethrin were again infested 6 months postintervention, and many uninfested sites became infested over the follow-up (Grijalva et al. 2011).

For *T. brasiliensis* in Ceará (northeast Brazil), residual spraying with SC deltamethrin or SC alpha-cypermethrin reduced house infestation or bug catch at 4–6 months post-application (more so in domiciles), and both metrics recovered baseline values approximately within 1 year postintervention (Diotaiuti et al. 2000; Bezerra et al. 2020). Also in Ceará, residual spraying with SC deltamethrin generated qualitatively similar results up to 1 year post-application, while a slow-release formulation of malathion outperformed other treatments in peridomiciles only (Oliveira Filho et al. 2000). For peridomestic *T. sordida* in Minas Gerais (southeast Brazil), bug population size almost completely recovered its preintervention levels 1 year after spraying with deltamethrin (Diotaiuti et al. 1998).

The available evidence supports that "a thorough application of appropriate pyrethroid insecticide will eliminate any domestic population of Triatominae" (Schofield 2000). This statement is conditional on applications made by trained spraymen who follow rigorous procedures in areas without pyrethroid resistance and does not apply to peridomestic (outdoor) structures where pyrethroids are rapidly degraded.

Effects on Secondary Triatomine Species A long-standing concern of vector control programs has been whether the decline or transient suppression of the target domestic vector may allow the niche expansion and colonization of human habitations by secondary triatomine species (e.g., Schofield and Dias 1999; Guhl et al. 2009). In two rural areas of the Argentine Chaco, community-wide pyrethroid spraying and selective retreatment targeting *T. infestans* reduced both the house infestation and relative abundance of *T. garciabesi* and *T. sordida* and the likelihood

of future infestation over a 10- and 3-year period, respectively (Rodríguez-Planes et al. 2016, 2020). Both species displayed fast recovery after interventions and marked seasonal fluctuations, but they failed to colonize human habitations despite the virtual elimination of the target vector. This generalized pattern suggests that other mechanisms may prevent domestic colonization by triatomine species that typically occupy peridomestic or sylvatic habitats.

Program Effectiveness Key to the effectiveness of residual insecticide spraying is the implementation strategy, which derives from the degree of organization, operational capacity, and accumulated experience of triatomine control programs. An insecticide-based control strategy involves the availability of a public health service with adequate levels of organization, trained personnel, infrastructure (e.g., vehicles for field transportation), funding appropriations (fuel, per diems), spraying gear (including safety equipment), supply of insecticides and spare parts, and organizational arrangements at various administrative levels. The functionality of this multicomponent system can be easily undermined if any of its components is missing.

Vector control operations have usually been organized in three successive phases. The preparatory phase involves administrative organization, training, and mapping of the affected areas selected for intervention. The attack phase includes spraying with insecticides all houses (full or "blanket" coverage) in two rounds separated by 6–12 months and selective retreatment of infested dwellings when the community-wide house infestation prevalence is below a predefined level (e.g., 5% in the Southern Cone countries targeting *T. infestans*). In endemic rural settings, villages and houses are treated under the rules of continuity (in time) and contiguity (in space) (Levy et al. 2010).

The surveillance phase entails monitoring of house reinfestation using adequate methods and selective retreatment of any infested house unit if the goal is vector control, as for *T. dimidiata* or *T. brasiliensis*, or of all houses within the community if the goal is regional elimination, as for *T. infestans* in Brazil or *R. prolixus* in Central America. In the latter case, the sole finding of an infested house after community-wide spraying determined the need to respray all houses in the village. Selective house treatment is indicated for intrusive triatomine species (e.g., Acevedo et al. 2000). Preventive spraying (i.e., treatment in the absence of infestation) has not played any role in routine program operations.

The outcomes of large-scale triatomine control programs throughout Latin America provide a solid line of evidence on the long-term effectiveness of house spraying with pyrethroid insecticides. The pioneering elimination of *T. infestans* from the State of São Paulo occurred in a context of large-scale economic, social, and landscape change (Wanderley 1993). The elimination goal was subsequently achieved by several other states in Brazil (Dias 2002). The Brazilian case and the regional elimination of *R. prolixus* from Central America (Hashimoto and Schofield 2012) attest to the success of well-established vector control programs targeting introduced vector species with no sylvatic foci. Similarly, the effectiveness of a single application of pyrethroids in a large-scale urban campaign against *T. infes-*

tans in Arequipa city (Peru) was estimated at 98.7% in the absence of insecticide resistance (Barbu et al. 2014). For other species and settings, especially in resource-constrained rural areas, triatomine control has often been substantially less effective. The effectiveness of an intervention protocol may vary widely between rural areas within the same municipality (Gurevitz et al. 2013; Provecho et al. 2017; Gaspe et al. 2018).

Large-scale program interventions sometimes reported vector control failures (i.e., under-average effectiveness) in the absence of pyrethroid resistance. Failures may originate from poor quality of the active ingredient and insecticide formulation; inappropriate or uncalibrated spray equipment; faulty dilution or spray technique; hard water; and lack of community engagement, which may interfere with insecticide applications or reduce the levels of treatment coverage (Vassena et al. 2007). Most of these factors are manageable via strict adherence to standards of good practice. Lack of exhaustive coverage of suitable triatomine habitats may be related to terrain, weather, habitat complexity, household mobility, and closed houses impeding access for insecticide application. Failure to spray peridomestic structures (perhaps to save labor or insecticide) may leave untreated foci or habitats where triatomines find a safe harbor. Passive transport of triatomines and persistent house invasion from external sources are not considered true vector control failures of insecticide-based programs, though they jeopardize vector elimination efforts.

Community-Based Vector Control The issue of recurrent house infestation (reinfestation) posed serious challenges to decentralized vector control programs operating in remote rural areas (Segura 2002) and still does. One way forward involved training and engaging the affected communities and primary healthcare system in vector control efforts (Chuit et al. 1992). Large-scale implementation of this strategy in a hyperendemic district (Moreno, Santiago del Estero) over a decade immediately reduced the incidence of *symptomatic* acute cases of Chagas disease and the prevalence of domestic infestation (to <20%) but had less impact on peridomestic infestation levels (Vazquez-Prokopec et al. 2009). In practice, the initial goal of vector elimination was replaced by acute disease prevention or reduction of the incidence of infection. A closer monitoring of infestation patterns in five rural villages of Moreno showed a strong connection between domestic and peridomestic foci of *T. infestans* under selective insecticide treatments conducted by householders over a 5-year period (Cecere et al. 2019).

The Reinfestation Process In endemic rural settings of the Argentine Chaco without pyrethroid resistance, domestic infestations with *T. infestans* were apparently suppressed for 1–2 years after a single community-wide residual spraying with pyrethroids applied by professional spraymen followed by no other control action (Fig. 2, Gürtler et al. 2007). This apparent success, in part true in domestic premises and in part derived from the difficulties associated with perceiving and detecting low-density infestations, favored the discontinuation of vector control actions. Bugs may require some time to arrive from elsewhere and recolonize the area depending on the geographic extent of treatment coverage, type of setting, and frequency of

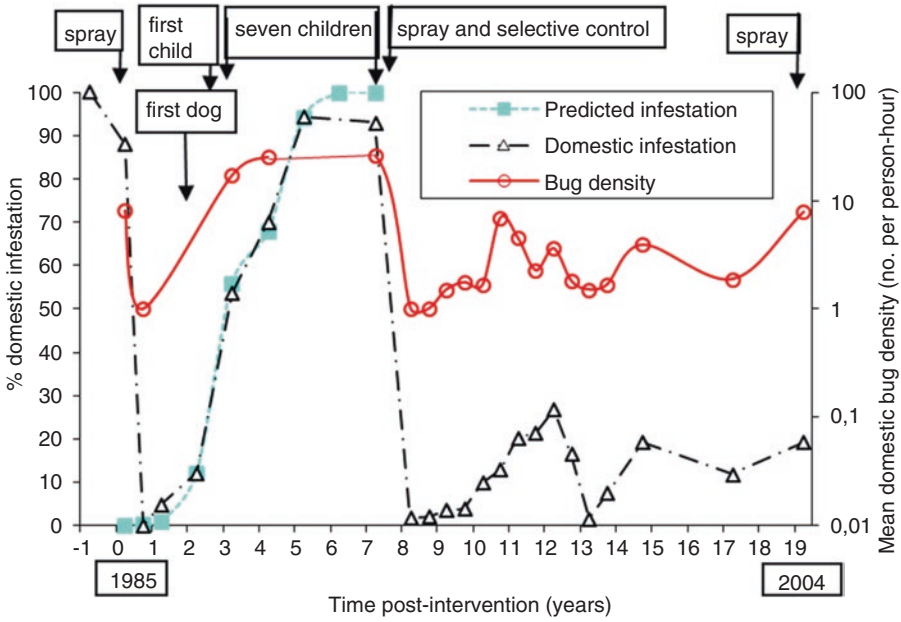

Fig. 2 Prevalence of domestic infestation (observed and predicted by a logistic model) and mean domestic density of *T. infestans* after two community-wide campaigns including residual insecticide spraying, Amamá and neighboring villages, 1984–2006. (Reproduced from Gürtler et al. 2007)

bug sources (domestic, peridomestic, and sylvatic). In the absence of vector surveillance and control, a single heavily infested house or peridomestic structure generated a village-wide reinfestation over 3–5 years postintervention (Fig. 2), with spatial clustering of new foci occurring within ~500 m of the putative source (Cecere et al. 2004, 2006b). These foci further propagated and caused new human infections with *T. cruzi* (Gürtler et al. 2005). Clustering distances are related to the vector's average flight dispersal range (see chapter "Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma Cruzi* in Domestic Habitats") and inform on where new secondary foci may appear.

A relevant question is whether the effectiveness of residual spraying with pyrethroids depends on local bug abundance. The postintervention prevalence of domestic or peridomestic infestation increased with increasing abundance of *T. infestans* before spraying in four rural areas (Gürtler et al. 1994, 2004; Cecere et al. 2002, 2006a; Gurevitz et al. 2013). Figure 1 shows the outcome of a district-wide randomized trial in which the fraction of infested sites postintervention steadily increased with preintervention bug abundance for each of four treatments with pyrethroids applied with manual or compression sprayers. These results strongly support that the effectiveness of a single application of pyrethroids decreases as local bug abundance increases, and by inference, they link treatment effectiveness to environmen-

tal and socioeconomic determinants of house infestation (see chapter "Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma Cruzi* in Domestic Habitats"). In this and other similar examples, the large reinfestation rates recorded in peridomestic structures may be explained by the inverse temperature dependency of pyrethroids and their reduced insecticidal activity outdoors and not by pyrethroid resistance. Moreover, the spatial extent, diversity, and number of structures contained in rural peridomiciles may hinder spraying operations and reduce effective coverage, thus creating a plethora of refugia and a de facto bug reservoir where genetic variability is preserved.

Resurgence of peridomestic infestations (recrudescence) frequently occurred after control interventions conducted in semi-arid rural areas in the absence of any pyrethroid resistance. Relevant examples include *T. sordida*, *T. brasiliensis* and *T. pseudomaculata* in Brazil (Oliveira Filho 1995; Diotaiuti et al. 1998, 2000); *T. dimidiata* in Central America (Nakagawa et al. 2003; Dumonteil et al. 2004), and *T. pallidipennis* in Mexico (Ramsey et al. 2003).

Sources of Reinfestation Romaña (1963) apparently was the first to identify the key question on the putative origins of triatomine bugs that appeared after full-coverage insecticide spraying. Recast in current terms the relevant alternatives are whether they are (i) survivors or the offspring of previously existing bugs (i.e., residual foci) or (ii) immigrants from untreated local houses (dispersing actively or passively) or from external foci (i.e., through passive transport from other villages or from sylvatic foci). This distinction is relevant because addressing the first mechanism would require improved application procedures, higher insecticide doses, or more effective insecticides, whereas passive transport from nearby villages calls for increased geographic coverage of insecticide spraying (Schofield 2000). All the abovementioned mechanisms are not mutually exclusive and may concur to a variable extent.

The potential sources of reinfestants differ between species of Triatominae and settings (see Sect. 2). Most of the *T. infestans* foci detected after a citywide insecticide campaign in Arequipa were attributed to nonparticipating households, i.e., nontreated houses (Barbu et al. 2014). By contrast, microsatellite-based studies revealed intense gene flow between sylvatic and domestic populations of *T. infestans* in three separate locations across the inter-Andean valleys of Bolivia (Brenière et al. 2013).

The consensus at the outset of the Southern Cone Initiative was that house reinfestation with *T. infestans* would occur from (i) domestic foci and involve local survivors, which in some cases would be "due to unusual construction features such as the dense roofs of brushwood and packed earth of some houses in the Chaco region," (ii) sylvatic foci (only in central Bolivia), and (iii) nontarget triatomine species (Schofield and Dias 1999, p. 20). Reinfestation from peridomestic foci was not mentioned, probably linked to the misleading notion that "*T. infestans* exclusively colonises intradomiciliary space" (Schmunis et al. 1996). In practice, the peridomestic foci of *T. infestans* have long been known in the Argentine Chaco and adjacent regions (e.g., Soler et al. 1977), though apparently they were less relevant in Brazil, where other secondary triatomine species took prominence (Dias 1991,

p 82). Whether the abovementioned misconceptions combined with semantic issues on the meaning of "domestic" affected decision-making and implementation of interventions in the most affected region remains a matter for speculation.

Pioneering studies using biochemical markers and traditional morphometry suggested that reinfestant *T. infestans* most likely were survivors from preintervention bug populations in Bolivia (Dujardin et al. 1996, 1997a, b). The frequent appearance of *peridomestic* bug colonies shortly after village-wide residual spraying with pyrethroids in the Argentine dry Chaco supported the notion that reinfestants were survivors or the offspring of triatomines existing prior to insecticide spraying, i.e., residual foci (Cecere et al. 1997). This hypothesis gained further momentum when several pieces of evidence collected in each of two distant rural areas were taken together: prespray and postspray local bug abundance and stage structure, spatial distribution of reinfestant foci, and duration of residual insecticide activity (Gürtler et al. 2004; Cecere et al. 2004, 2006b). Moreover, comparison of prespray and postspray *T. infestans* populations using 10 microsatelli loci added further weight to the hypothesis of residual foci (Marcet 2009).

Subsequent studies in this area revealed the occurrence of at least six sylvatic foci of *T. infestans* in the periphery of three infested rural villages: microsatellite markers and wing geometric morphometry showed that sylvatic specimens were genetically indistinguishable from their domestic or peridomestic counterparts, suggesting they were feral derivatives or spillovers (Ceballos et al. 2011). Vector control actions affected the genetic structure of *T. infestans* populations but not allele richness and genetic diversity (Marcet et al. 2008). Bug populations from communities under recurrent insecticide spraying were highly structured (including significant genetic differentiation between neighboring house compounds), whereas populations under sporadic spraying were more homogeneous and conformed one genetic cluster. Whether spraying with pyrethroids enhanced the flight dispersal of *T. infestans* toward untreated habitats remains unclear.

Elsewhere in western Argentina, a microsatellite-based analysis showed that almost all *T. infestans* collected in domestic and peridomestic sites of individual houses shared ancestry, supporting the hypothesis of substantial gene flow between peridomestic and domestic populations (Pérez de Rosas et al. 2013). In the Argentine humid Chaco, both wing geometric morphometry (Gaspe et al. 2013) and microsatellite markers (Piccinali et al. 2018) supported that most of the *T. infestans* foci detected over the first year postintervention were residual foci. However, microsatellite markers also showed that a few of the reinfestants could not be assigned to any of the preintervention source populations (Piccinali et al. 2018). This is hardly surprising for domestic triatomine species whose spatial spread is frequently mediated by inadvertent transport in luggage or goods: bug sources may be quite distant to the points of destination (Piccinali et al. 2010).

For *R. prolixus* in Venezuela, both wing geometric morphometry (Feliciangeli et al. 2007) and microsatellite markers (Fitzpatrick et al. 2008) provided consistent evidence of bug dispersal between sylvatic and domestic habitats, and in consistency with insecticide trials (Sánchez-Martin et al. 2006), they showed that palm tree bug populations posed significant threats of house reinfestation after insecticide

spraying campaigns. Gómez-Núñez (1969) had reached similar conclusions by using radioactive-tagged wild *R. prolixus* 40 years before. For *T. dimidiata* in Guatemala, vector kinship and population-genetic analysis using single nucleotide polymorphic markers showed that house reinfestation after selective spraying with pyrethroids recovered fast from local survivors (residual foci) and in-migrants from neighboring houses or villages (Cahan et al. 2019; Stevens and Dorn 2017).

Epidemiological Impact While the immediate goals of triatomine control programs are to reduce or suppress house infestation (presence-absence) and abundance, or even eliminate the vector species from a region, their ultimate goal is to prevent the appearance of new vector-borne infections (i.e., halt parasite transmission) and future human disease. Therefore, the relevant metrics to establish the degree of progress are transmission-related indices, such as those produced by large-scale, sequential surveys that measure the seroprevalence of *T. cruzi* infection in well-defined age groups (e.g., children under 5 or 10 years of age). Repeated cross-sectional serosurveys conducted over an extended period provide the necessary information to estimate incidence rates (force of infection) and define whether vector-borne transmission has been interrupted or not (Hoff et al. 1985). The appearance or notification of *symptomatic* acute cases of Chagas disease is an insensitive transmission index because they are a small fraction of all new cases and frequently are not detected or reported. Other appropriate transmission-related indices closely linked to incidence of human infection, such as the relative abundance of *T. cruzi*-infected triatomines in domiciles, have rarely been used to assess the effectiveness of triatomine control actions and should be employed more widely.

When vector surveillance and control actions are sustained over time, the resulting age-seroprevalence curve expresses the impact of such interventions by flattening over the early age classes and by shifting the ascending arm to the right. This broad pattern was repeatedly verified by sequential age-structured serosurveys both in well-defined areas (Fig. 3) and in large-scale program operations, as in Venezuela (Aché and Matos 2001, Feliciangeli et al. 2003) and Colombia (Cucunubá et al. 2017). Conversely, new human infections with *T. cruzi* occurred when vector control operations reduced but failed to suppress domestic infestations in a sustainable manner, for example, in a *P. megistus*-infested rural area of Bahia (northeast Brazil) repeatedly treated with bendiocarb or malathion (Sherlock and Piesman 1984; Piesman et al. 1985). Estimating threshold infected-vector densities or house infestation rates that would eventually lead to the interruption of vector-borne transmission (or achieve a desired target level) is fraught with many empirical difficulties (see chapter "Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma Cruzi* in Domestic Habitats"). Threshold infected-vector densities will most likely differ between triatomine species and locations, and the spatial scale at which they should be measured (household, village, district) is unclear. The issue becomes irrelevant when the goal is vector elimination.

Other major assessments of program impacts have been made. In Argentina, cohorts of 18- or 21-year-old military recruits surveyed for anti-*T. cruzi* antibodies (totaling 2.1 million men) revealed a striking drop in nationwide seroprevalence

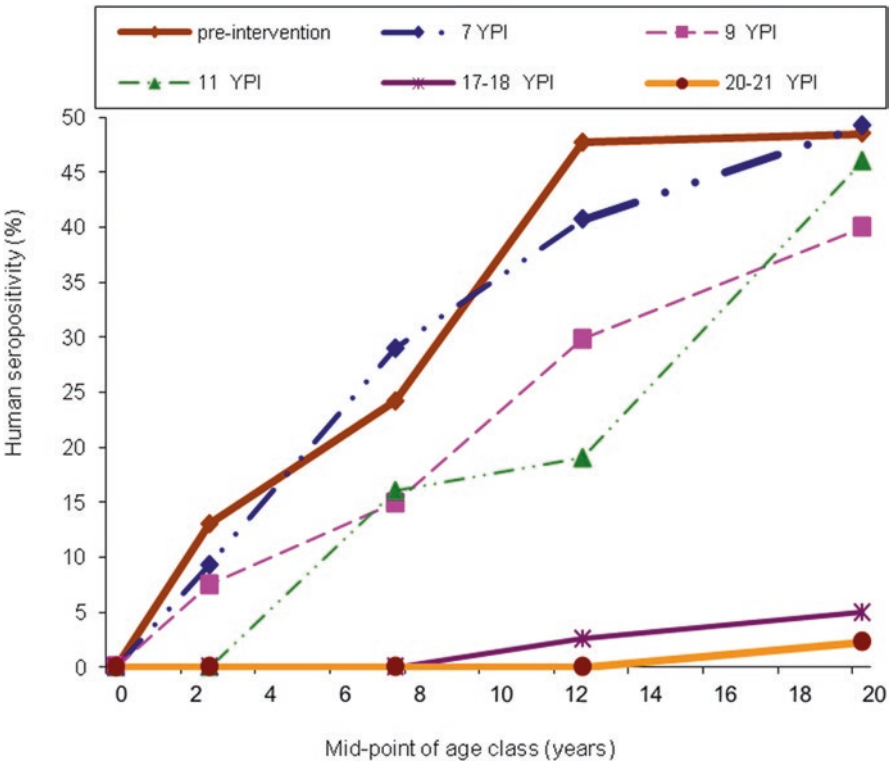

Fig. 3 Age-specific prevalence of seropositivity *T. cruzi* in humans <25 years of age in Amamá and four neighboring villages, 1984–2006, before initial interventions in 1985, and during sustained surveillance with selective control actions 1992–2006. (Reproduced from Gürtler et al. 2007)

from 10.1% in 1965–1969 to 5.8% in 1981 and 1.9% in 1993 (Segura et al. 2000). The rate of decline over time was heterogeneous among and within regions and was attributed to the intensity of past insecticide spraying campaigns. In Brazil, a nationwide serosurvey of nearly 237,000 schoolchildren aged 7–14 years conducted over 1989–1999 showed marginal seropositivity rates (0.14%) of *T. cruzi* and a strong positive correlation between domestic infestation with any triatomine species and child infection (Dias 2002, p. 224). A new nationwide serosurvey of children aged less than 5 years detected an even lower seroprevalence (0.03%) over 2001–2008 (Ostermayer et al. 2011). While these assessments demonstrate the steady decline in the seroprevalence of human *T. cruzi* infection in connection to the sustained operations of triatomine control programs, they also occurred in the context of major regional changes in landscape, socioeconomic development, and rural-to-urban migration. For example, major changes in rainfall patterns allowed the expansion of intensified agriculture in several sections of the Southern Cone including the southern Chaco (Hoyos et al. 2013). Such land-use changes frequently pushed the rural

poor to peri-urban or urban settlements where house infestation may become established (Bayer et al. 2009; Gaspe et al. 2020).

Pyrethroid Resistance High levels of resistance to pyrethroid insecticides associated with control failures of *T. infestans* emerged in Salta (northwest Argentina, on the border with Bolivia) by the late 1990s (Zaidenberg 2012; Picollo et al. 2005). The local vector control program had detected unusually high levels of house infestation soon after houses were sprayed with pyrethroids in 1998. Although there were some reports of reduced susceptibility to pyrethroids in *R. prolixus* (in Venezuela) and *T. infestans* (in southern Brazil), none of them had been connected to vector control failures (Vassena et al. 2000; Mougabure-Cueto and Picollo 2015). Other pyrethroid-resistance foci of *T. infestans* were subsequently detected throughout Bolivia (Lardeux et al. 2010; Depickère et al. 2012; Gomez et al. 2014), and their occurrence was linked to selected environmental variables (Gomez et al. 2016).

This suite of findings and heightened awareness prompted the detection of the first moderate-resistance focus of *T. infestans* associated with vector control failures in northeastern Argentina by 2008 (Gurevitz et al. 2012). The new focus occurred in a province (Chaco) with a sparse history of pyrethroid-spraying campaigns against *T. infestans* and preannounced the emergence of several high-resistant foci in neighboring rural areas (e.g., Fronza et al. 2016).

Other triatomine species have displayed reduced susceptibility to pyrethroids elsewhere, including *T. sordida* from Minas Gerais (Pessoa et al. 2015); *Panstrongylus geniculatus* collected in Santander, Colombia (Torres et al. 2013); and *T. mazzotti* and *T. longipennis* from Mexico, which revealed mutations in the *kdr* gene (Dávila-Barboza et al. 2019). Recently, wild *R. prolixus* bugs collected from oil and native palm trees in Casanare (Colombia) displayed significant pyrethroid resistance and increased levels of detoxifying enzyme activity, possibly related to pyrethroid applications targeting oil palm pests (Calderón et al. 2020). Whether such levels of reduced susceptibility cause vector control failures is a key question across triatomine species and settings.

Pyrethroid-resistant populations of *T. infestans* have shown three well-known mechanisms: reduced penetration, enhanced metabolism (mediated by esterases and cytochrome P450 monooxygenases), and modified site of action (likely involved in cases of high resistance) (Mougabure-Cueto and Picollo 2015; see chapter in this book). This suggested that pyrethroid resistance may have developed independently through different evolutionary processes. All highly resistant populations of *T. infestans* from the Argentine-Bolivian border and from Chaco province carried either one of two mutations in the *kdr* gene (Sierra et al. 2016). But pyrethroid-resistant triatomines incur in fitness costs, including reduced rates of development, fecundity, and defecation (Germano and Picollo 2015; Lobbia et al. 2018). Whether the joint effects of these and other modified traits may effectively affect parasite transmission is unknown.

The putative occurrence of pyrethroid resistance in sylvatic populations of *T. infestans* across Bolivia (presumably not exposed to any house spraying with

insecticide) attracted wide interest in the search for the ancestral phenotype of susceptibility to pyrethroids. All four sylvatic populations of *T. infestans* from Cochabamba and Potosi were resistant to deltamethrin, with mean resistance ratios (RR_{50}) ranging from 1.9 to 11.9 (Acevedo et al. 2011). In Mataral (Cochabamba), domestic and sylvatic *T. infestans* had similar levels of pyrethroid resistance exceeding tenfold those of the susceptible reference strain (Acevedo et al. 2011). Additional efforts throughout Bolivia revealed only 1 of 12 sylvatic foci of *T. infestans* with reduced susceptibility to pyrethroids (Depickère et al. 2012), 2 sylvatic foci in Cochabamba with low pyrethroid resistance (RR_{50} = 0.62–4.24), and 3 fully susceptible sylvatic foci in the Bolivian Chaco (Gomez et al. 2014). In summary, the majority of sylvatic populations of *T. infestans* examined in Bolivia so far were susceptible to pyrethroids. Taken together with evidence showing intense gene flow between sylvatic and domestic *T. infestans* in Bolivia (Brenière et al. 2013), the most parsimonious explanation to the rather few pyrethroid-resistant sylvatic foci detected so far is that they may be feral derivatives of domestic or peridomestic populations.

All pyrethroid-resistant populations of *T. infestans* were cross-resistant to other pyrethroids and susceptible to organophosphorus and carbamate insecticides (Mougabure-Cueto and Picollo 2015). In the absence of more appropriate alternatives, vector control programs resorted to the application of fenitrothion, malathion, or bendiocarb despite their less favorable properties. In the laboratory, fenitrothion increased the mortality rate of pyrethroid-resistant *T. infestans* for a relatively short period depending on the specific substrate (Germano et al. 2014). In field operations conducted over a decade in Salta, suppression of pyrethroid-resistant *T. infestans* populations required several rounds of 40% WP fenitrothion combined with insecticide fumigant canisters or 100% EC malathion (alone or combined), whereas houses sprayed with 80% WP bendiocarb showed greater infestation rates post-application (Zaidenberg 2012). In this area, vector responses to control actions differed widely between geographic sections, and spray coverage was frequently suboptimal because of closed or vacant houses.

Pyrethroid-resistant populations of *T. infestans* persist in some of the affected areas of the Argentine Chaco (e.g., Fronza et al. 2016; Enriquez et al. 2020). These resistant populations should be closely monitored and targeted for prompt elimination to prevent further spread. A similar policy should be adopted with pyrethroid-susceptible residual populations of *T. infestans* in Rio Grande do Sul and Bahia states, Brazil (Belisário et al. 2017). Because of growing concerns on domestic spraying with organophosphorus and carbamate insecticides (WHO 2016) and their banning in some countries (e.g., Argentina since 2017), there is a clear need of new alternative insecticides or tactics. A thorough compilation of triatomine populations screened for pyrethroid resistance does not show any apparent trend in the frequency of resistant populations over the last decades (Flores-Ferrer et al. 2018).

Corollary Pyrethroids are highly effective but they are no silver bullets. The available evidence indicates that residual spraying with SC pyrethroids may leave a variable number of residual foci, most of which occur outdoors. Domestic and

peridomestic foci of *T. infestans* are well connected through dispersal, and both may function as sources for reinfestation (Gürtler et al. 2014; Cecere et al. 2019). *Triatoma dimidiata* in Central America and *R. prolixus* in Venezuela and Colombia may function in an analogous manner (Stevens and Dorn 2017). In the presence of productive foci (peridomestic or sylvatic) and houses susceptible to bug invasion and colonization, the elimination of domestic infestations is a transient state. Keeping the vector out of domestic premises would require sustained surveillance and recurrent, effective control actions. In the absence of such actions, the notion of decoupled (independent) domestic and peridomestic populations of *T. infestans* and other major vector species with similar or broader habitat use patterns has little supportive evidence, if any, and should be abandoned. Similarly, for triatomine species that colonize domestic premises, their peridomestic and sylvatic foci usually differ in several key aspects relevant to vector control. Unlike peridomestic foci, sylvatic foci typically involve small-sized bug colonies dominated by stochastic processes, are more distant to domestic premises (with distance acting as a partial barrier), and are virtually inaccessible to current control actions. We conclude that peridomestic foci and effective vector surveillance at low bug densities remain the Achilles' heel of triatomine control programs.

Marsden (1984, p. 859) nicely described the problem: "Insecticides alone will never solve the problem. They must be associated with a program of vigilance for detection of residual and fresh infestation of houses by bugs." In his vision, program success depends on the dominant bug species and strength of public health services; house improvement was "…a useful measure in the right circumstances." Both statements are still valid. In real-life practice, multiple spraying rounds were required to knock down house infestation rates in the most affected areas, e.g., for *T. dimidiata* in Central America (Hashimoto et al. 2006) and *T. infestans* in the Gran Chaco region (Gurevitz et al. 2013). The documented effectiveness of large-scale triatomine control programs is inherently linked to sustained efforts and due attention to effective vector surveillance and timely control responses rather than to sporadic spraying campaigns paying more attention to public visibility than to sustained disease prevention. Program organization (Hashimoto and Yoshioka 2012) and sustained political commitment are therefore essential (Schofield and Dias 1999; Dias 2002; Gorla and Hashimoto 2017).

Insecticidal Paints and Fumigant Canisters

Insecticidal paints, mostly based on a polyvinyl acetate emulsion of malathion or other organophosphorus compounds developed over the 1980s, showed long-lasting efficacy indoors and were cost-effective in relation to residual spraying with pyrethroid insecticides (Oliveira Filho 1989). Disposable fumigant canisters release an insecticidal smoke after the fuse is lit. They were deemed suitable for nonprofes-

sional, community-based control of domestic infestations with triatomines and to curtail the operational costs of traditional vector surveillance (Zerba 1988; Rozendaal 1997). Fumigant canisters were used as part of a primary healthcare-based control strategy (Chuit et al. 1992) and as a sensitive triatomine detection method because of its rapid knockdown effects (Gürtler et al. 1993, 1999). The latest version of fumigant canisters contains beta-cypermethrin, permethrin, and dichlorvos and has a residual activity <8 days (Dias and Zerba 2001). Therefore, two to three monthly applications of two cans per 30 m^3 were recommended to suppress established domestic infestations. Prior to treatment of mud-and-thatch rural houses with fumigant canisters, eaves and other openings should be closed to block or slow down the escape of fumes.

A multicountry field trial compared the effectiveness of a slow-release malathion-based paint applied to domestic and peridomestic structures; fumigant canisters combined with house spraying applied to domestic and peridomestic structures, respectively; and traditional residual spraying with insecticides in both structures (Oliveira Filho 1997). The insecticidal paint performed significantly better than other treatments when assessed 6 months post-application, whereas residual insecticide spraying performed better than fumigant canisters although not significantly so. Householders in Honduras predominantly viewed residual spraying with pyrethroids as the most effective treatment, whereas insecticidal paints were valued less favorably because of adherence- and application-related issues (Montes et al. 1999).

A new generation of insecticidal paints (including a microencapsulated formulation of 1.5% chlorpyrifos, 1.5% diazinon and pyriproxyfen) showed extended lethal residual activity against pyrethroid-resistant *T. infestans* over 34 months post-application in the Bolivian Chaco (Alarico et al. 2010). In most of the houses, the insecticidal paint was applied after householders plastered the walls of domestic premises, thus creating a mixed intervention. This insecticidal paint showed immediate and long-lasting impacts on domestic and peridomestic populations of *T. infestans* over 32 months post-application in the same region (Gorla et al. 2015). In an experimental setup simulating natural conditions, surfaces treated with this insecticidal paint induced significantly greater mortality of fifth-instar nymphs of *T. infestans* than those either sprayed with deltamethrin or not treated (Maloney et al. 2013). A related insecticidal paint containing pyrethroids and pyriproxyfen applied to various types of bricks exerted substantial lethal effects on *T. infestans* nymphs over at least 1 year post-application (Amelotti et al. 2009).

The new paint formulations may include different active ingredients with repellent and lethal effects on vector species transmitting different pathogens (Schiøler et al. 2016). Microencapsulated insecticides are protected from rapid environmental degradation and afford prolonged residual effects, especially outdoors. The main sources of concern are prolonged exposure to chemical compounds that present potential health hazards, the added expense of insecticidal paints, and whether they can be applied to the typical mud-and-thatch houses that prevail in endemic rural areas.

Xenointoxication and Insecticide-Impregnated Materials

Xenointoxication is a targeted vector control strategy that involves the application of pesticides on nonhuman hosts to kill the bugs that contact or blood-feed on them (Romaña and Abalos 1948). In one of its early applications in a heavily infested facility, treatment of 50 chickens with BHC caused massive mortality of *T. infestans*, reduction of bug population size, and induced various toxic effects in chickens (Rocha e Silva et al. 1969).

Deltamethrin-impregnated dog collars experimentally reduced the feeding success and blood engorgement of *T. infestans* and eliminated bug populations contained in closed huts kept under natural conditions (Reithinger et al. 2005, 2006). In contrast, spot-on fipronil administered to dogs under the same conditions was not able to suppress experimental bug populations (Gürtler et al. 2009). A spot-on formulation of imidacloprid applied to pigeons induced large mortality on pyrethroid-resistant *T. infestans* up to 7 days posttreatment (Carvajal et al. 2014). A pour-on formulation of cypermethrin administered to goats and chickens reduced the blood intake of *T. infestans* and killed them over approximately 1 month post-application (Amelotti et al. 2012, 2014). Other spot-on formulations of beta-cypermethrin, combined with pyriproxyfen or not, also reduced bug population size in closed chicken coops relative to non-treated control units, whereas no effects were detected in pyriproxyfen-treated replicates (Juan et al. 2013).

Oral administration of fluralaner (a novel isoxazoline ectoparasiticide) to dogs showed promising results. In canine feed-through assays, a single dose killed first- to fourth-instar nymphs of *T. infestans* up to 51 days posttreatment (Loza et al. 2017). In a field-based trial, fluralaner was effective against both pyrethroid-susceptible and resistant fifth-instar nymphs of *T. infestans* up to 120 days post-application and exerted no anti-feeding effects (Laiño et al. 2019).

Pyrethroid-impregnated fabrics (Wood et al. 1999), bednets, and curtains (Herber and Kroeger 2003; Kroeger et al. 2003) exerted repellent effects and increased the mortality rate of *T. infestans* and *Rhodnius* sp. in experimental and field trials. Deltamethrin-impregnated netting covering guinea pig enclosures substantially reduced their rate of infestation with *T. infestans* in Arequipa, Peru (Levy et al. 2008). Insecticide-treated durable wall lining attached to inner house walls would offer a new supplementary tool for domestic triatomine control conditional on favorable efficacy trials, lack of toxicity derived from prolonged exposure to chemical compounds, and solving feasibility and economic constraints (Messenger and Rowland 2017).

Although all of the abovementioned compounds or delivery mechanisms provided proof-of-principle evidence, so far none apparently outperformed the average cost-effectiveness of residual house spraying with pyrethroids against susceptible triatomines.

5.2 *Housing Improvement*

Housing quality and domestic infestations with triatomines are closely connected since the early days of Carlos Chagas (Dias and Schofield 2004). Rural houses in Chagas disease endemic areas throughout Latin America are traditionally built using mud, sticks, wooden poles, brushwood, and palm leaves. Construction materials combined with their degree of maintenance determine the availability of refuges for triatomines both in human sleeping quarters and peridomestic habitats (see chapter "Eco-Epidemiology of Vector-Borne Transmission of *Trypanosoma Cruzi* in Domestic Habitats"). Plastering walls with appropriate soil-cement mixtures and replacing thatched roofs with ceramic tiles or sheets of corrugated metal or fiber cement (with ceilings for insulation) will eliminate hiding places and reduce domestic bug infestations and increase both bug exposure to insecticides and detectability. The quantitative impact of these conditions on bug abundance is closely related to the quality of housing improvement, including the type of ceiling and subsequent maintenance. Household goods (e.g., beds and hanging bags or clothes) provide important habitats for *T. infestans* (Cecere et al. 1998; Lardeux et al. 2015). In the absence of adequate bug control responses by householders, even well built, urban-type houses may become infested (e.g., Marsden 1984; Gaspe et al. 2018, Fig. S2). Nonetheless, such domestic bug populations may not reach the large densities recorded in endemic rural areas (Gaspe et al. 2020).

Traditional mud-and-thatch houses largely differ in the construction materials used, state of repair, and their suitability for triatomine bugs. In Santiago del Estero (northwest Argentina), thatched roofs made of a long-leaved grass ("simbol") arranged in compact bundles and topped with packed earth left few refugia for triatomines; they were highly valued by local residents because "it brought no pests." When "simbol" roofs were combined with plastered mud walls and nonprofessional use of domestic insecticides, domestic bug densities were significantly lower than in other typical rural houses (Cecere et al. 1998), and dwellers had lower infection rates with *T. cruzi* (Gürtler et al. 1998). Mud-and-thatch houses provide adequate insulation against temperature extremes in subtropical areas, unlike the small urban-type houses frequently provided by rural housing programs. Householders frequently kept both types of houses and used them according to prevailing weather, thus defeating one of the goals of housing improvement programs.

Rural housing programs have usually acted independently from triatomine control programs except in a few countries and regions (e.g., Venezuela since the 1950s, southern Bolivia, and Guatemala) (Briceño-León 1990; Guillén et al. 1997; Monroy et al. 2009; Alarico et al. 2010). Rural housing programs depend on rural development policies, tenured land ownership, and access to credit. Changes in land use combined with the steady trend of rural-to-urban migration have added further obstacles to such investment. Although householders may provide the labor needed to fix or replace a dilapidated hut using well-established construction techniques (e.g., Rozendaal 1997), cost is a major limitation and adequate training is required to develop the necessary skills. Thus, the overall rate of replacement of inadequate

rural houses has been quite slow even in stable regions, the more so when there are chronic resource constraints and employment dwindles.

Peridomestic animal shelters and grain storage constructions are also targets for structural improvement and may impact directly on triatomine infestation and domestic animal health. In semi-arid areas of the Gran Chaco, the brushwood-made fences of goat or sheep corrals are usually heavily infested with several triatomine species (Soler et al. 1977; Gürtler al. 2004; Cohen et al. 2017). Replacing brushwood with wire fencing virtually eliminates all bug refuges and substantially reduces the surface that would need to be sprayed with insecticides. In an experimental intervention trial, improvement of goat corrals reduced site infestation and bug abundance and increased goat production in western Argentina (Gorla et al. 2013). Similarly, replacing small chicken nests made of mud with appropriate materials would affect one of the main habitats of *T. infestans* in the Argentine Chaco (Gurevitz et al. 2013; Gaspe et al. 2015; 2020). Community-based improvement of peridomestic structures may be the strategic response to the pervasive problem of peridomestic triatomine populations. Such endeavor requires intersectoral collaboration between rural development agencies, housing, and health ministries.

Effects of Housing Improvement Only a few intervention trials have assessed the sole or joint effects of housing improvement and residual insecticide spraying on domestic reinfestation, and most of them were inspired by ecohealth-based interventions. In a large-scale intervention program conducted in Venezuela (1977–1985), domestic house infestation with *R. prolixus* was significantly reduced from 55.9% to 0% after nearly 5 years of improving or replacing palm-leaved roofs and palm or mud walls in seven villages from Trujillo State, where the vector was resistant to dieldrin and BHC (OPS 1998). Human incidence rates also dropped to one-third of reference levels. Conversely, in the absence of housing improvement, house spraying with insecticides applied irregularly in five villages from Portuguesa State (with BHC-susceptible triatomines) reduced domestic infestations nonsignificantly from 10.2% to 5.8%, while the incidence rates of human infection tripled the reference level (OPS 1998).

In Bolivia, a long-term intervention program including insecticide spraying, plastering of walls, and improvement of chicken and rabbit coops greatly reduced domestic and peridomestic infestations with *T. infestans* and child infection rates, but there was no comparison group (Cassab et al. 1999). Similarly, an ecohealth-based intervention package including plastering walls with appropriate local materials, house cleaning activities, and removing chickens and dogs to outdoor areas in two villages substantially decreased domestic infestation and bug abundance in reference to the outcomes recorded in two control villages under an annual insecticide spraying schedule (Lardeux et al. 2015).

In Paraguay, traditional residual spraying of WP lambda-cyhalothrin alone reduced domestic infestations with *T. infestans* to 5% of baseline levels by 18 months post-application, whereas housing improvement alone or combined with residual insecticide spraying achieved lower relative reductions and was less cost-effective (Rojas de Arias et al. 1999). Three children residing in houses subjected to house

modification alone seroconverted for *T. cruzi*; the origins of these new infections remained uncertain. In northwest Argentina, plastering of walls before spraying with SC deltamethrin slightly reduced domiciliary infestation and colonization rates over the subsequent 3 years compared with community-wide spraying alone, while domestic bug infection rates plummeted in both treatment groups (Cecere et al. 1999, 2002).

In Guatemala, a 3-year intervention trial evaluated the effects on domestic and peridomestic infestation with *T. dimidiata* of plastering walls with locally available materials and sand (performed by householders) combined with information activities, versus traditional spraying with WP deltamethrin in four villages (Monroy et al. 2009). Although both strategies substantially reduced bug infection rates and domestic bug abundance (with residual spraying showing larger effects), none of them suppressed infestations and bug infection with *T. cruzi*. Peridomestic bug abundance increased approximately threefold after environmental measures and was halved after residual insecticide spraying. Subsequent interventions targeting earth floors and chicken coops in one of the study villages kept domestic infestation rates at low levels over an additional period of 3 years (Lucero et al. 2013).

Also in Guatemala, a cluster-randomized controlled trial tested a combined intervention package including educational workshops, improved insecticide spraying procedures, implementation of rodent control actions, and training in organic waste management and productive household activities, relative to traditional residual insecticide spraying (De Urioste-Stone et al. 2015). Although both intervention arms were equally efficacious in reducing preintervention domestic infestations over a 2-year period, the combined treatment significantly reduced rat infestations, human and rodent-vector contact, and domestic bug infection rates in reference to the control arm. In Yucatan (Mexico), insecticide-impregnated curtains alone or combined with untreated window screens reduced house infestation with intrusive *T. dimidiata* (Ferral et al. 2010; Waleckx et al. 2015).

These intervention trials include a complex combination of study designs, settings, triatomine species, tactics, and mechanisms of delivery. Taken together, they do not provide a unique conclusion in reference to the relative efficacy of house improvement and residual insecticide spraying and their joint effects on domestic reinfestation and incidence of human infection. The large-scale intervention program conducted in Venezuela produced compelling evidence of the impact of house improvement on domestic and peridomestic infestation, but parasite transmission was reduced and not fully interrupted. Interventions targeting a fraction of the house compound (i.e., walls or domestic premises) left many alternative refuges for triatomines. The order of the interventions may affect the final outcome: the initial community-wide residual spraying is expected to substantially reduce bug population size and leave few sources, on which subsequent house modifications may act on additively or multiplicatively. As an example of how to expedite joint interventions smoothly, the municipality of Camiri (Bolivian Chaco) provided householders with construction materials (de bono) and required them to finish house improvements before providing them the insecticidal paint. Our on-site observations and those of other researchers elsewhere in the region (Gorla et al.

2015) suggest that the esthetic value of renewed, color-painted traditional houses (widely hailed) acted as a powerful catalyzer of intervention uptake.

A systematic review of six carefully selected articles concluded that there was weak evidence on whether modifications of housing structures had an impact on triatomine control and found strong evidence on indoor residual spraying being the most powerful intervention (Horstick and Runge-Ranzinger 2018). Our overview also shows that the evidence base for house improvement effects is weak though suggestive of protective effects. This does not undermine the need of improving access to a healthy housing environment (for which there is undisputed evidence) regardless of its immediate effects on triatomine infestation. Housing improvement and other multisectoral environmental measures directed to prevent house infestation with triatomines have not been exploited to its full potential and may play an important role for integrated vector management across diseases (WHO 2017).

5.3 Biological and Genetic Control

Triatomine bugs have numerous natural enemies, including predators (especially Reduviidae bugs and spiders), egg parasitoids (e.g., *Telenomus costalimai* and *Telenomus fariai* (Scelionidae) and *Ooencyrtus venatorious* (Encyrtidae)), parasitic mites, and various insect pathogens (Coscarón et al. 1999). The prevalence of eggs parasitized by the abovementioned parasitoids usually ranged from 1 to 10%, but in some sylvatic habitats it reached 37%. Biological control of Triatominae using egg parasitoids was field trialed with discouraging results (Rabinovich 1985). The notion of biocontrol has little applicability for triatomine species with public health significance, for which the goal is to suppress domestic populations rather than to establish a low-density equilibrium between insect hosts and parasitoids.

Following the detection of two fungal entomopathogens (*Beauveria bassiana* and *Paecilomyces lilacinus*), a wide survey of pathogens in *T. infestans* and a few other triatomine species throughout Argentina found the widespread occurrence of a Triatoma picornavirus (mean prevalence, 9.66%) and low frequencies (0.44%) of the pathogenic trypanosomatidae *Blastocrithidia triatomae* (Marti et al. 2009). The use of *B. bassiana* formulated as a biopesticide, combined with a bug attractant in a shelter box, substantially increased the mortality rate and reduced the fecundity of pyrethroid-resistant *T. infestans* in closed experimental huts (Forlani et al. 2015).

Genetic methods involve either direct transformation of an insect genome via mobile DNA elements (transgenesis) or expression of gene products in the host insect via genetically transformed symbiotic bacteria that interfere with vector competence without affecting insect fitness (paratransgenesis) (Hurwitz et al. 2011). This approach rests on several requirements and remains in the experimental stage.

6 Current Challenges and Opportunities

Residual house spraying with insecticide has proven successful in suppressing or reducing house infestations with major triatomine vectors and interrupting parasite transmission. Residual insecticide spraying has also been difficult to sustain when local or regional elimination was not achieved. Major obstacles to sustainability of vector and disease control efforts include lack of long-lasting state policies, economic swings and political instability, recurrent operating costs (e.g., insecticide), ill-defined program goals and metrics to measure progress, environmental or human health concerns, and dwindling community participation (Gürtler 2009).

Although the same methods and insecticides have been in use over the last 40 years, they continue to be effective in most circumstances if applied properly. Identifying their limitations is the path to maximizing effectiveness and selecting complementary tactics. In the past, failure to meet program goals can largely be attributed to poor implementation of vector control operations (e.g., substandard spatial or temporal coverage, inconsistent vector surveillance and response), weakened organization and political support in conjunction with economic hardship rather than pyrethroid resistance. These issues are especially important in remote rural areas, where transportation costs and accessibility pose additional constraints and public visibility is marginal. In the context of the growing circulation of mosquito-borne viruses throughout Latin America over the last three decades, fewer resources have been allocated to triatomine control.

Program managers frequently lack updated infestation or disease data to assist the decision-making process and prioritize interventions. This is an area where potential gains may be obtained by using modern geospatial tools and decision support systems. Spatial aggregation of house or village infestation and human infection is generalized across triatomine species and settings (e.g., Levy et al. 2009; Vazquez-Prokopec et al. 2009; Fernández et al. 2019; Cohen et al. 2018) and so are geographic sections where the outcome of routine procedures is below expectations (e.g., Hashimoto et al. 2006). These sections should be prioritized for intensified control efforts and disease risk reduction.

The pace at which new pyrethroid-resistant foci are discovered has remained stable so far, but prospects are uncertain. Suspect vector control failures should be investigated in detail, and broad monitoring of pyrethroid resistance is required. Because very few public health insecticides have been developed over the last 50 years (Hemingway 2017), there is a great need of new insecticides and formulations that can cope with peridomestic triatomine populations and pyrethroid-resistant foci in a cost-effective way.

Integrated vector and disease management may be the key to sustainability in the affected areas (WHO 2012). Linking vector management with diagnosis and etiologic treatment of the affected populations may stimulate long-term community commitment and sustainability of disease control efforts.

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Insecticide Resistance in Triatomines

Gastón Mougabure-Cueto and María Inés Picollo

Abstract The control of Chagas disease vectors has been based principally on spraying dwellings with insecticides. This strategy reduced the geographic range and infestation prevalence of major vectors leading to the interruption of disease transmission in several areas from endemic regions. However, triatomine survival after spraying pyrethroid insecticides, mainly in the case of *Triatoma infestans* (Hemiptera: Reduviidae), has become more frequent in the last two decades. Insecticide resistance emerges as one main explanation for these chemical control failures. This chapter reviews the evolution of insecticide resistance in triatomines. Resistance to pyrethroids was first detected in *T. infestans* in the 1990s. But, it was only in the 2000s that resistance associated with control failures was described for the latter species in Argentina and Bolivia. Different resistant profiles were demonstrated for *T. infestans* suggesting that resistant foci originated independently. The main resistance mechanisms (i.e., enhanced detoxification, target-site modifications, and reduced penetration) were described for this species. Resistance to deltamethrin in *T. infestans* was shown to be controlled by an autosomal and incompletely dominant character. The resistance evolving in *T. infestans* from the Chaco ecoregion would be associated with different pleiotropic effects of the genes that confer resistance. Moreover, environmental variables linked to temperature and precipitation would explain part of the distribution of resistant populations of *T. infestans* in some endemic areas of the Chaco ecoregion. Finally, the possible resistance management strategies for triatomines are discussed.

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1 Introduction

The development of resistance to insecticides has been demonstrated in most pest insect species exposed to chemical control (McKenzie 1996). Insecticide resistance is an evolutionary phenomenon through which insecticide use acts as a selective force that favors preexisting features conferring resistance to part of the insect population. This selection reduces the susceptibility to the applied insecticide that was previously effective for controlling individuals of this species. This is because resistant individuals survive after exposure to the insecticide and transmit their genetic background to their offspring (Fig. 1).

According to the Insecticide Resistance Action Committee (IRAC), resistance to insecticide is "a heritable change in the susceptibility of an insect population that is reflected in the repeated failure of the insecticide application to achieve the expected level of control when used according to the label recommendation for that species."

The early detection of resistance in a population exposed to an insecticide is of considerable importance to avoid the development of high ratios of resistance (RR:

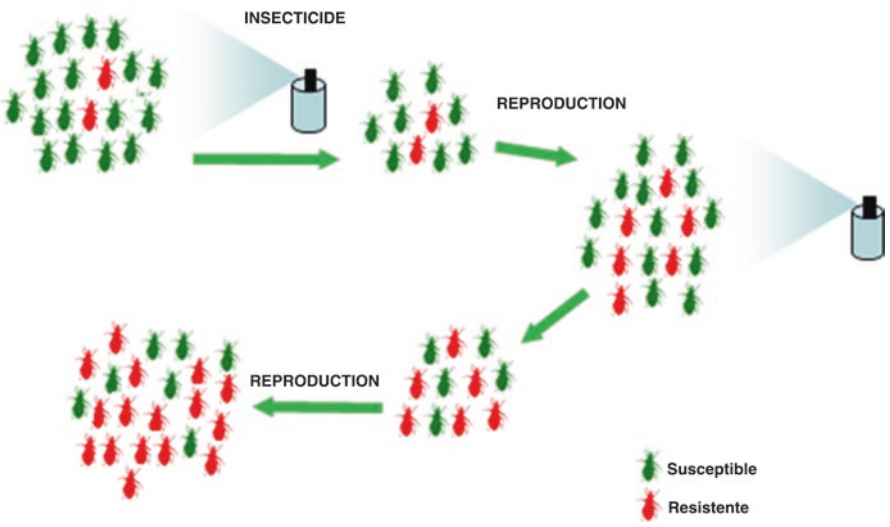

Fig. 1 Evolution of the resistance to an insecticide in an insect population
Insecticide application mainly eliminates susceptible individuals. The most resistant individuals survive and pass the resistant genetic character to the offspring. After several generations, the proportion of resistant individuals was increased, and the effectiveness of the insecticide in the population was reduced

LD_{50}-resistant population/LD_{50}-susceptible population) and to elaborate alternative control strategies adequate for that resistant population. The development of standardized tests fit to specific insect pests is of considerable importance for resistance detection activities. Additionally, resistance surveillance has been facilitated by incorporating the "discriminating dose" (DD) concept, namely, the use of a dose of an insecticide that is lethal to susceptible individuals, but allows the survival of resistant ones (ffrench-Constant and Roush 1990). The definition of such discriminating dose is based on a prior accurate determination of the dose-mortality curve and the estimation of the lethal dose for 99% (LD_{99}) of the exposed individuals of a susceptible population of the species.

The discriminating doses for *Triatoma infestans* (Klug) and *Rhodnius prolixus* (Stahl) were established as the lethal dose for 99% of the tested individuals according to the World Health Organization protocol. The protocol indicates that first instars (5–7 days old, mean weight 1,3 mg) starved since eclosion are treated by topical application on the dorsal abdomen with 0.2 microliter of the insecticide diluted in acetone. The mortality rates of control and treated insects are then recorded after 24 h according to their ability to reach the border of a 7 cm filter paper disc when released in its center, alone or after mechanical stimulation (WHO 1994). The use of this protocol allows comparing results obtained by different laboratories studying triatomine insecticide resistance. As an example, this protocol was used to assess the resistance to deltamethrin status of various Bolivian and Argentinian field populations of *T. infestans* (Lardeux et al. 2010; Germano et al. 2013). Two reviews on the resistance to insecticides in vectors of Chagas disease have been published in the last decade (Mougabure-Cueto and Picollo 2015; Pessoa et al. 2015).

2 Populations Resistant to Insecticides

Triatomine control efforts have been mainly based on the application of insecticides. This has allowed achieving an important reduction in the domestic infestation and vectorial transmission of Chagas disease in Latin America. The first insecticides used to control triatomines were organochlorines such as hexachlorobenzene (HCB) and dieldrin (Zerba 1999). Later, the carbamates propoxur and bendiocarb were introduced in the 1960s mainly because of their proven efficacy and ovicidal properties. Subsequently, the organophosphorus malathion was recommended for the control of Chagas vectors in the 1970s. Finally, various pyrethroid insecticides were proven to be effective during the 1980s for controlling these vectors (Mougabure-Cueto and Picollo 2015).

Early studies evaluating field populations of *T. infestans* from Argentina that had been exposed to vector control campaigns detected growing percentages of deltamethrin-resistant populations (from 29% in 1997 to 40% in 2002, respectively, for Vassena et al. 2000 and González-Audino et al. 2004). These populations showed

low levels of resistance (resistant ratios <10) that did not correlate with field control failures.

It was only with the advent of the new millennium that a decreased effectiveness was reported for the chemical control of triatomines in several areas of Argentina, Bolivia, and Colombia. High resistance to pyrethroid insecticides associated with ineffective field treatments was detected in 2002 for *T. infestans* in the San Martín department of Salta province in northern Argentina and Yacuiba, Sucre, and Mataral in southern and central Bolivia (Picollo et al. 2005). The resistance ratios for these pyrethroid-resistant populations of *T. infestans* ranged from 20 to 100.

High prevalence of infestation by *T. dimidiata* (Latreille) was reported in deltamethrin-sprayed dwellings from Colombia (Reyes et al. 2007). Besides, the presence of *R. prolixus* from sylvatic populations was reported for sprayed dwellings from an extensive geographic area of Colombia (Llanos Orientales) and Venezuela, where a high percentage of houses remained positive 1 year after spraying fenitrothion and deltamethrin (Sánchez and Jesús 2006; Angulo et al. 2006). Moreover, moderate (RR <100) and low (RR <10) levels of resistance to lambda-cyhalothrin and fenitrothion, respectively, were determined for a field population of *Panstrongylus geniculatus* (Latreille) collected in 2012 in the Chorreras, Capitanejo municipality, Santander department, Colombia (Torres et al. 2013).

Susceptibility to deltamethrin, malathion, and bendiocarb was characterized for 50 populations of *T. infestans* sampled in Bolivian human dwellings a decade ago (Lardeux et al. 2010). These populations were resistant to deltamethrin (RR: from 6 to 491), while none of them exhibited significant resistance to malathion or bendiocarb. Additionally, high resistance to deltamethrin (RR: from 25.6 to 54.7) was reported in domestic and sylvatic *T. infestans* collected in 2014 in four communities of the Municipality of Torotoro, Potosí department, Bolivia. Interestingly, different resistance values were estimated in insects from peridomiciliary structures of the same dwelling, indicating that this phenomenon is complex both at the community and microgeographical levels (Espinoza Echeverria et al. 2018).

All these investigations established that resistance to deltamethrin is high and widespread in Bolivia. In contrast, high deltamethrin resistance in Argentina seemed to be initially restricted to Salta province, in the northwestern area of the country (Germano et al. 2010a). Infestation by *T. infestans* after repeated spraying with pyrethroids was more recently reported in several rural areas of Chaco province in northern Argentina. High resistance to deltamethrin was assessed in the Güemes department of the Chaco province, in localities such as El Malá (Carvajal et al. 2012), La Esperanza (Germano et al. 2013, 2014), El Juramento (Sierra et al. 2016), Pampa Argentina, and El Asustado (Fronza et al. 2016). The levels of deltamethrin resistance assessed in these localities were the highest found so far (RR >1000). In Pampa del Indio, another area of the Chaco province located 103 km from the abovementioned ones, reinfestation due to the presence of resistant insects (RR: from 4.47 to 11.50) was reported after initial spraying with deltamethrin (Gurevitz et al. 2012) (Fig. 2).

In summary, resistance to insecticides was demonstrated for different triatomine species in several areas of their geographical distribution in the last 20 years. This

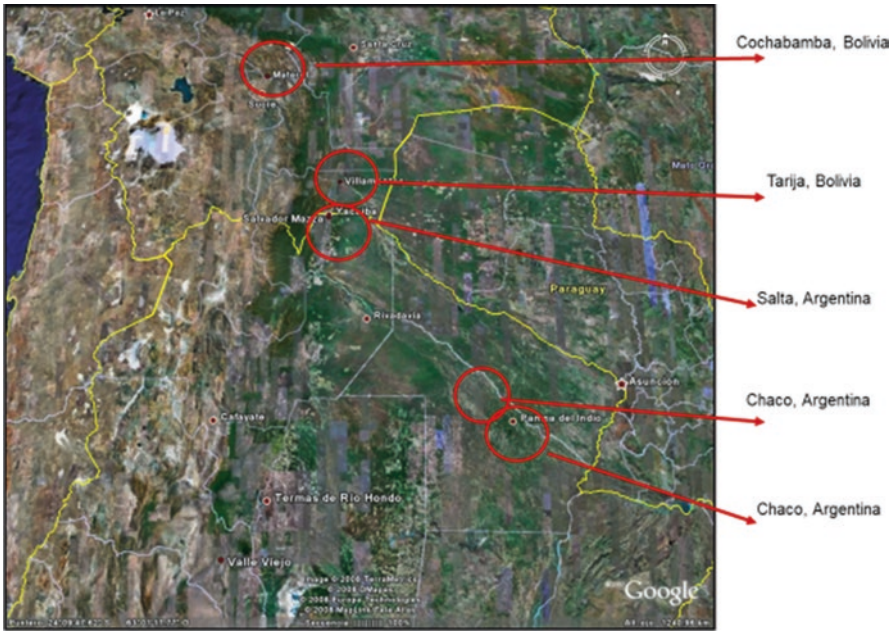

Fig. 2 Geographical distribution of high deltamethrin-resistant populations of *T. infestans* from Bolivia and Argentina
Areas where high resistance to pyrethroid insecticides was associated with ineffective field chemical treatment for *T. infestans* from Bolivia (Cochabamba and Tarija departments) and northern Argentina (Salta and Chaco provinces)

canceled the former prevailing assumption that there was a low probability of occurrence of insecticide resistance in triatomines due to an apparently low genetic variability in domiciliated species. At present, it is known that triatomine species showed rich genetic variability through its distribution range, and it has been demonstrated that this phenomenon is more widespread than was previously assumed.

3 Resistance Profiles

Several studies demonstrated that pyrethroid-resistant populations exhibit different toxicological profiles when exposed to the same chemical class. Deltamethrin-resistant *T infestans* populations from the Salta province, Argentina (Salvador Mazza, La Toma, El Chorro, and El Sauzal), were shown to be resistant to other pyrethroids (beta-cypermethrin, beta-cyfluthrin, and lambda-cyhalothrin), but not to fenitrothion (organophosphorus insecticide), bendiocarb (carbamate), or fipronil (phenylpyrazolez insecticide) (Toloza et al. 2008; Santo-Orihuela et al. 2008). Germano et al. (2012) defined three resistant profiles according to the toxicological and biochemical characteristics of resistant populations from Acambuco (Argentina)

and Entre Ríos and Mataral (Bolivia). The Acambuco profile exhibited moderate deltamethrin resistance in nymphs and eggs (RR: 32.5 and 28.6, respectively) and increased detoxifying metabolism as a resistance mechanism through enhanced pyrethroid-esterase activity. The Entre Ríos profile exhibited high deltamethrin resistance (RR: 173.8) in nymphs and moderate in eggs (RR: 39.1), low resistance to fipronil (RR: 12.4), and increased pyrethroid-esterase activity. Finally, the Mataral profile exhibited low deltamethrin resistance in nymphs and eggs (RRs: 17.4 and 8.4, respectively), increased pyrethroid-esterase activity, and moderate level of resistance to fipronil. Obviously, the characterization of these three profiles does not permit the generalization of three resistance forms. Besides, different levels of susceptibility to deltamethrin and fipronil were reported for sylvatic *T. infestans* that had never been exposed to chemical control. This suggests that populations from different geographic areas have naturally different toxicological responses to insecticides (Roca-Acevedo et al. 2011; Gomez et al. 2014).

The differences in resistance profile and susceptibility to insecticides reported for field populations of *T. infestans* suggest independent evolutionary processes based on insecticide pressures, genetic variation of natural populations, and local environmental factors. It is possible to propose that insecticide pressure was not the main cause generating these profiles because vector control campaigns in the studied areas had been sparse, and fipronil was never used as triatomicide. This hypothesis was later evaluated by analyzing the effects of environmental and spraying factors on the distribution of *T. infestans*-resistant populations (Gomez et al. 2014; Fronza et al. 2019) (see Sect. 7 in this chapter).

In the search of alternatives to control deltamethrin-resistant *T. infestans*, several insecticides with different modes of action were evaluated on a highly resistant population from Chaco, Argentina (see Sect. 8 for details). As an outcome, fenitrothion and imidacloprid were found effective against both susceptible and resistant populations, becoming control alternatives (Carvajal et al. 2012). Fenitrothion formulated as wettable powder showed high effectiveness for vector field control (Zaidemberg 2012). For imidacloprid, only a spot-on formulation applied to pigeons in a laboratory bioassay was shown effective against pyrethroid-resistant *T. infestans* that fed on treated pigeons (Carvajal et al. 2014).

4 Resistance Mechanisms

Several biochemical/physiological processes modify the individual susceptibility determining the resistant phenotype; these are called resistance mechanisms. Furthermore, resistant insects can exhibit more than one of these mechanisms at same time (McKenzie 1996). These mechanisms are alterations of the toxicokinetic and toxicodynamic steps occurring during the insect- insecticide interaction which determine the degree to which the toxicant can damage the organism (i.e., the toxicity) or, what is the same, the degree to which the organism is damaged by the toxicant (i.e., the susceptibility) (ffrench-Constant 2013). Toxicokinetics describes what

happens to the toxicant when it makes contact with the insect and once inside it and involves the absorption, distribution, metabolism, and excretion processes. Toxicodynamics describe the molecular interaction between the toxicant and the molecular target, also known as the site of action, which will trigger the toxic response (Hodgson and Levi 1997). The main mechanisms altering insecticide toxicity are enhanced enzymatic detoxification, altered target sites, and reduced cuticular penetration. The three mechanisms have been described for triatomine pyrethroid-resistant populations (Mougabure-Cueto and Picollo 2015).

Enhanced Detoxification Insects have evolved various detoxification mechanisms to survive to natural toxins. These mechanisms can allow them to overcome insecticides by a rapid enzymatic detoxification. Pyrethroids are mainly cleaved by monooxygenase activity and esterase-mediated hydrolysis. Monooxygenase or P450 enzyme activity leading generally to a detoxification of the molecule can be involved in the metabolism of virtually all insecticides. Esterases deserve main attention because they can hydrolyze ester bonds present in chemicals that are extensively used by vector control programs (Roush and Tabashnik 1990; McKenzie 1996). For some insects, detoxification is so active that the insecticide does not reach its molecular target before being metabolized and degraded by the enzymes.

Increased monooxygenase and pyrethroid-esterase activity was assessed in pyrethroid-resistant *T. infestans* populations from the Salta and La Rioja provinces of Argentina and in the Yacuiba department of Bolivia (Santo-Orihuela et al. 2008). This increased enzymatic activity allows insects a faster breakdown of the insecticide to nontoxic compounds. Enhanced insecticide metabolization was also demonstrated for late developing embryos of these resistant populations. The most significant increase in enzymatic activity was observed for pyrethroid esterase, which grew steadily throughout the embryonic development of both susceptible and resistant populations, even though being always higher in resistant embryos (Roca-Acevedo et al. 2013, 2015). Recently, Grosso et al. (2016), Traverso et al. (2017), and Dulbecco et al. (2018) showed overexpression of P450 genes belonging to the CYP4 clade in deltamethrin-resistant *T. infestans* from Argentina. However, it is worth to mention that both inhibiting the detoxifying enzymes and RNAi-mediated gene silencing of CYP4s led to slight increases or did not affect the susceptibility to deltamethrin of resistant insects, suggesting a secondary role for these enzymes in mediating resistance (Picollo et al. 2005; Dulbecco et al. 2018).

Target-Site Modifications Altered target site is another mechanism conferring pyrethroid resistance in triatomines. Pyrethroids exert their insecticidal action on the insect nervous system by modifying the normal function of voltage-gated sodium channels in the membranes of neurons. In resistant insects, these effects on the nervous system are reduced due to the presence of point mutations in the sodium channel gene. Most resistance-conferring mutations affecting voltage-gated sodium channels are located in domain II of this protein, particularly the region between transmembrane segments IV and VI (IIS4-IIS6 region) (Soderlund and Knipple 2003).

Fabro et al. (2012) cloned and sequenced the domain II of the *T. infestans* sodium channel based on the sequence of this domain in the *Rhodnius prolixus* para sodium channel. Thus, the presence of a resistance-conferring mutation (L1014F) was identified in a deltamethrin-resistant population (RR: 35.7) of Madrejones, a small locality of Salta province of Argentina. Later, a different new pyrethroid resistance-conferring mutation (L925I) was identified in a deltamethrin-resistant population of Mala, a small locality of the Chaco province of Argentina (RR: 1031). Interestingly, and unlike the L1014F mutation described for many insect species belonging to different orders, the L925I mutation seems to be exclusive of hemipterans (Capriotti et al. 2014). Recent studies demonstrated that both mutations were associated with two geographically differentiated foci of *T. infestans*-resistant populations: those on the Argentina-Bolivia border carrying L1014 mutation and those from the Argentinian Chaco province carrying L925I mutation (Sierra et al. 2016).

Reduced Cuticle Penetration The integument covers the insect externally. It is formed by the cuticle and the epidermis, playing essential protection roles to avoid desiccation, mechanical injury, and toxins. Indeed, the cuticle is the first and major barrier that insecticides should penetrate to reach target sites.

Penetration resistance refers to cuticular alterations that reduce the entry rate of toxic compounds within the insect body. This can be effected through either modifying the composition of the cuticle or increasing its thickness. These mechanisms prevent or delay reaching toxic levels of insecticide molecules at the target site in the nervous system. Moreover, the reduced rate of penetration allows detoxification enzymes more time to act multiplying their effectiveness in the degradation of the toxic compound (Roush and Tabashnik 1990).

Two mechanisms have been described to explain penetration resistance in triatomines, cuticular thickening and altered cuticular composition (Pedrini et al. 2009). In fact, the cuticle of *T. infestans* fourth-instar nymphs of deltamethrin-resistant populations from Salta and Chaco provinces (Argentina) was significantly thicker. The cuticle width revealed by scanning electron micrographs (SEM) of transversal sections of the second abdominal tergite was 32.1+/−5.9 micrometers and 17.8+/−5.4 micrometers for pyrethroid-resistant and pyrethroid-susceptible insects, respectively. Moreover, these resistant populations had an impressive increase in the amount of cuticular hydrocarbons (CHCs), 50% more CHCs, revealed by capillary gas chromatography coupled to mass spectrometry analyses. Since HC-free cuticles were more susceptible to insecticide penetration, it was proposed that CHC enrichment would delay the uptake of pyrethroids through the cuticle.

Although most of the detoxification metabolism of insects is considered to occur in the fat body, new members of the cytochrome P450 gene family (*CYP*) that belong to the highly genome-wide expanded *CYP3093A* and *CYP4EM* subfamilies were recently described in the integument of *R. prolixus* and *T. infestans.* A suite of CYP4-clan genes was overexpressed (1.7–3.8-fold) in the integument of deltamethrin-resistant *T. infestans* nymphs (*CYP3093A11* and *CYP4EM10*), as was evidenced by the biochemical determination of increased activity of the ethoxycoumarin-O-deethylase. These studies suggested a cytochrome P450-based

detoxification role of the integument in the deltamethrin-resistant insects (Dulbecco et al. 2018).

In summary, after more than two decades of studies, resistance to insecticides in triatomines is known to occur by a multiplicity of physiological-biochemical mechanisms that can act in an overlapping manner (i.e., enhanced metabolism, modified site of action, and reduced penetration).

5 Inheritance and Genetic Basis of Insecticide Resistance

The individual susceptibility is the expression of multiple biochemical and physiological processes that occur during the toxicokinetic and toxicodynamic phases of the insect-insecticide interaction, each one determined by genetic and environmental factors (Mougabure-Cueto and Sfara 2016). Due to variation in these factors, individual susceptibility is randomly distributed among the individuals of a population. This distribution is expressed as a symmetric sigmoid curve when the proportion of responding individuals (dead) is plotted against insecticide dose, i.e., the cumulative normal distribution of susceptibility (Hewlett and Plackett 1978). It is on the genetic variation that underlies the distribution of susceptibility where the insecticide selection pressure operates. The consequence is a shift of the dose-response curve toward higher doses. If the active dose of insecticide falls within this distribution, a polygenic-based resistance is expected because the selected individuals are part of a distribution partially determined by many genes (i.e., the most likely scenario in laboratory selection). On the other hand, if the dose of insecticide is higher than the maximum dose of the susceptibility distribution, the resistance is expected to be determined by one or a few genes because the selected individuals probably carry rare mutations that places them outside the original distribution (i.e., the most likely scenario in field selection) (McKenzie 1996; ffrench-Constant 2013).

Cardozo et al. (2010) showed that the resistance of insects from Salvador Mazza (Argentina) would be controlled by more than one gene and, presumably, at least three. This is consistent with the studies described above (see Sect. 4 in this chapter) that showed that different resistance mechanisms (i.e., two families of detoxifying enzymes, two mutations in the sodium channel, and alterations in the cuticle) were present in *T. infestans* even within the same population, which shows that resistance to deltamethrin is determined by more than one gene. Moreover, Cardozo et al. (2010) and Germano et al. (2010b) showed that deltamethrin resistance in *T. infestans* is an autosomal and incomplete dominant character. Finally, Bustamante Gomez et al. (2015) showed that the resistance ratio increased more than 20 times in 2 generations after selection with deltamethrin and estimated a realized heritability (h^2) equal to 0.37, suggesting that resistance is an additive and cumulative factor.

6 Pleiotropic Effects of the Insecticide Resistance

The genetic alterations that generate the individual resistance to an insecticide can have pleiotropic effects on different physiological processes (other than the toxicological processes involved as resistance mechanisms) with possible consequences at ecological and evolutionary levels (McKenzie 1996; Kliot and Ghanim 2012). In addition, in vector insects, these effects can have direct consequences on disease transmission by modifying the vectorial capacity of the insect (Rivero et al. 2010). Pleiotropic effects can occur due to a detrimental modification of physiological processes involving genes that carry resistant mutations or genes linked to them or by the maintenance of the resistant phenotype that reduces the energy available for other processes (Rivero et al. 2010; Kliot and Ghanim 2012). The pleiotropic effects may be selectively neutral or may have positive or negative consequences with respect to the adaptation of the resistant insects to the natural environment (i.e., without the toxicant) (Mougabure-Cueto and Picollo 2015). The negative consequences are those that are more likely to be represented in a resistant population and are referred to as biological or adaptive costs of resistance, while positive consequences are involved in the evolution of tolerant populations as adaptations (Lobbia et al. 2018). In addition to the evolutionary implications, this comparison in adaptive terms has a practical importance for vector control and resistance management strategies. In the case of adaptive costs, the proportion of resistant insects is expected to decrease when chemical control is discontinued. Alternatively, it is not expected to decrease when pleiotropic effects are adaptively positive (Mougabure-Cueto and Picollo 2015).

The few studies comparing the performance of different biological processes between susceptible and resistant triatomine phenotypes were carried out on pyrethroid-resistant *T. infestans* from Argentina. Germano and Picollo (2015) investigated possible modifications in the ontogenetic development and reproductive potential of resistant insects from Aguaray village (Salta province, Argentina). The study demonstrated reproductive costs, expressed as a lower fecundity, and developmental alterations, expressed as shorter second and third nymph stages and a larger fifth stage. The authors also suggested a maternal effect since these alterations were observed in resistant females and their progeny regardless of the toxicological phenotype of the male. Lobbia et al. (2018) studied the alterations in the excretion/defecation pattern in resistant *T. infestans* from La Rinconada and El Asustado villages located in the high resistance focus of the Chaco province (Argentina). The resistant insects began to defecate later, defecated less, and showed a lower proportion of defecating individuals compared with susceptible insects during the first hour after feeding. The authors suggested that the alterations could generate an adaptive cost in the natural environment and that the resistant insects would have a lower vector competence than susceptible ones. Lobbia et al. (2019a) studied the effect of resistance on the reproductive efficiency after dispersal in *T. infestans* from El Asustado village (Chaco province, Argentina). In non-dispersed control insects, the resistant females showed lower reproductive efficiency than susceptible females.

However, the dispersed resistant females showed a higher reproductive efficiency compared to the dispersed susceptible females and compared to the non-dispersed resistant females. The authors suggested that the resistant insects probably carry an adaptive cost when both toxicological phenotypes do not disperse but, if both phenotypes disperse, the resistant insects would have an adaptive advantage over the susceptible ones. Finally, Lobbia et al. (2019b) investigated the effect of resistance on the dispersal capacity of *T. infestans* from El Asustado village (Chaco province, Argentina). The resistant insects showed a lower number of dispersal events, a lower proportion of dispersed individuals, and less exit and entry events from/to experimental shelters. This lower dispersal capacity associated with the resistance could reduce the colonization and reinfestation capabilities and possibly the relevance of the resistant insects as vectors.

The insects studied in the latter reports came from the Argentine Chaco ecoregion. Therefore, the evolution of resistance in this region might be associated with a complex expression of pleiotropic effects of genes that confer resistance or genes linked to them (Lobbia et al. 2019b). This scenario could be explained in part by the multiple resistance mechanisms detected in the different resistant foci (Germano et al. 2012; Mougabure-Cueto and Picollo 2015; Sierra et al. 2016).

7 Environmental Factors Associated with Insecticide Resistance

As emerges from the definition, insecticide resistance evolves as a consequence of the continued use of insecticides. The insecticide is the main determinant, acting as a selection agent. However, environmental variables, both from the natural environment and associated with anthropic activities, may influence the evolution of resistance. Variables that affect the effectivity of the insecticide will determine the position of the dose used in the dose-response curve (i.e., the proportion of individuals of the population that is affected by the insecticide). Thus, this will determine the intensity of selection pressure. This context frames the possible role of the peridomestic structures of the rural dwellings in the evolution of insecticide resistance in triatomines. The intricate construction and diverse materials used for these structures hinder the uniform application of the insecticide, exposing insects to variable doses. Besides, due to their presence in open air, the surfaces of these structures expose insecticide molecules to degradation by light and heat (Cecere et al. 2004; Gürtler et al. 2004). In this way, the effective dose of an insecticide decays rapidly, allowing the survival of less susceptible individuals and the evolution of resistance (see Sect. 5). This scenario is different from what happens in the intradomicile. There, the insecticide lasts longer in its optimal dose (i.e., lethal for 100% of the susceptible individuals) and would only select the individuals located outside the natural distribution of susceptibilities of the population, if they were present (Mougabure-Cueto and Picollo 2015; Fronza et al. 2019). Thus, it is possible that

toxicological heterogeneity exists between both environments of a dwelling. The only study that compared *T. infestans* from domestic and peridomestic areas found no statistical association between toxicological status (resistant vs. susceptible) and original location of the insects (Germano et al. 2013).

In addition to their effect on the insecticide selection regime, environmental variables might be directly involved in a process related to the evolution of resistance: the evolution of tolerance to insecticides. Some environmental variables could select individuals carrying certain phenotypic attributes not involved with the interaction with the insecticide but determined by genes that simultaneously confer individual resistance or that are linked to resistant genes. In this situation, the frequency of resistant insects would increase in the natural environment without insecticide resulting in a population with low natural susceptibility, i.e., a tolerant population. The low susceptibilities to pyrethroids and the phenylpyrazole fipronil shown by sylvatic populations of *T. infestans* from Bolivia (Roca-Acevedo et al. 2011; Depickère et al. 2012; Gomez et al. 2014) have been interpreted as an indication of the existence of naturally tolerant populations (Roca-Acevedo et al. 2011; Mougabure-Cueto and Picollo 2015).

The evaluation of relative roles for environmental factors and variables linked to spraying with insecticides (e.g., frequency, coverage, etc.), as well as their possible interaction, has received little attention in studies about the evolution and distribution of resistance in triatomines. Bustamante Gomez et al. (2016) and Fronza et al. (2019) have studied whether environmental variables show an association with the distribution of pyrethroid-resistant *T. infestans* populations. Bustamante Gomez et al. (2016) carried out a bibliographic review of studies reporting pyrethroid resistance in the latter species and showed that resistance is a localized event associated with a combination of variables linked to temperature and precipitation in the area of the border between Argentina and Bolivia. The model developed by the authors allowed to describe the potential distribution of highly resistant populations, but did not detect 5 out of 13 populations in the department of Güemes from Chaco province (Argentina), the area where the highest resistance levels were reported for *T. infestans* (Carvajal et al. 2012; Sierra et al. 2016, Fronza et al. 2016). Alternatively, Fronza et al. (2019) focused their study on this area of very high resistance and high toxicological heterogeneity. These authors included bioclimatic statistics and information on chemical control actions as explanatory variables and showed that three indicators of temperature and precipitation are good descriptors for insecticide resistance. Furthermore, their models increased their explanatory power when village-size variables were added. The spraying variables did not contribute to explain toxicological heterogeneity, possibly due to the fact that the pressure with insecticides in the area had been homogeneous and executed with low frequency. The authors proposed that the environmental variables explain part of the resistance distribution because they modulate the selection pressure exerted by the insecticide (e.g., the temperature modifying the toxicity of pyrethroids and the precipitation affecting the availability of insecticide).

8 Management of Insecticide Resistance

The evolution of insecticide resistance leads to the failure of previously successful chemical control strategies. Therefore, resistance management strategies must be implemented. The main goal of these strategies is to initially detect resistance at the lowest level possible and interrupt the ongoing selection process. A complete resistance management strategy should monitor the toxicological profile of populations under chemical control, identify resistance mechanisms, develop alternative control strategies (i.e., alternative insecticides, biological control, etc.), and study the biological process underlying the evolution of resistance (i.e., population structure, dispersal capacity, costs of resistance, etc.) (Mougabure-Cueto and Picollo 2015). Before resistance reaches a level leading to field failures, and even before knowing the existence of resistance, the so-called resistance prevention tactics can be implemented (e.g., rotation of insecticides, use of refuges for susceptible individuals, etc.) (McKenzie 1996). However, such strategies only work if resistance genes are present in the population; if there are no resistance genes, their implementation is meaningless. These tactics are, in fact, methods that interrupt or delay the selection process, avoiding increases of the resistance level of the population.

The management of insecticide resistance in triatomines is determined and conditioned by their role as disease vectors and by the biological, social, cultural, and operational characteristics associated with Chagas disease and its control. The use of strategies that maintain susceptible individuals in the population is hampered because triatomines transmit a pathogen causing a human disease. The shortage of insecticides and formulations for the control of Chagas disease vectors does not allow the implementation of strategies such as molecule rotation or the use of mixtures of insecticides that belong to unrelated chemical classes. On the other hand, effective alternatives to the spraying of dwellings with residual insecticides have not yet been developed and implemented on a large scale. However, four main tools could be effective in controlling pyrethroid-resistant insects: (1) insecticide treatment of domestic animals acting as food sources for triatomines (Reithinger et al. 2005, 2006; Gürtler et al. 2009; Amelotti et al. 2009a, b, 2012; Juan et al. 2013; Carvajal et al. 2014; Dadé et al. 2014, 2017); (2) use of insecticidal paints based on microencapsulated formulations containing organophosphates or pyrethroids, together with an insect growth regulator (Dias and Jemmio 2008; Amelotti et al. 2009b; Alarico et al. 2010; Maloney et al. 2013); (3) entomopathogenic fungi, like *Beauveria bassiana*, as biological control agents (Luz et al. 1999, 2004; Pedrini et al. 2009; Forlani et al. 2011; Zumaquero-Rios et al. 2014); and (4) traps that combine structures that allow insects to enter but hinder their exit with physical or chemical stimuli that attract insects to such structures (Guerenstein et al. 1995; Guidobaldi and Guerenstein 2013; Mota et al. 2014). In turn, these baited traps can be combined with adhesive surfaces, insecticides, or some natural enemy so that they exert a control action (Pedrini et al. 2009). Future research is needed to assess the effectiveness of these alternatives on a large scale, as well as their safety to humans and the domestic and natural environment.

The organophosphates fenitrothion and malathion, and the carbamate bendiocarb (acetylcholinesterase inhibitors), are the current effective alternatives for the control of resistant foci of *T. infestans* in the context of traditional chemical control. In particular, resistant foci in Argentina have been controlled with fenitrothion or malathion (Zaidemberg 2012; Gurevitz et al. 2012; Germano et al. 2014), while bendiocarb was mainly used in Bolivia (Programa Nacional de Chagas 2009). However, the toxicological risk of these insecticides (greater than that of pyrethroids) and the quality and receptivity of their formulations by residents are the main reasons that promote the search for alternative insecticides. Several non-pyrethroid insecticides from different chemical groups have been evaluated on susceptible and deltamethrin-resistant *T. infestans* in laboratory assays. Amitraz (octopamine receptor agonist), flubendiamide (ryanodine receptor modulator), ivermectin (chloride channel activator), indoxacarb (voltage-dependent sodium channel blocker), and spinosad (nicotinic acetylcholine receptor allosteric activator) showed no lethal activity on either susceptible or resistant populations at the doses tested (Carvajal et al. 2012). Fipronil (GABA-gated chloride channel blocker) showed high activity against resistant populations from Argentina but was not effective against resistant populations from Bolivia (Toloza et al. 2008; Germano et al. 2010b). Finally, imidacloprid (nicotinic acetylcholine receptor agonist) showed high toxic potency against susceptible and resistant *T. infestans* (Carvajal et al. 2012). The latter results promote the development of an adequate formulation for Chagas vectors and its evaluation under field conditions.

In summary, current triatomine control is based on spraying pyrethroid insecticides (e.g., deltamethrin), mainly formulated as suspension concentrates, as long as no resistant foci are detected. As soon as a systematic pyrethroid application is implemented in an area, the toxicological (i.e., tracking the evolution of resistance) and genetic (i.e., determining the field frequency of resistant alleles) monitoring of bug populations are necessary actions that allow the detection of resistance in early stages of evolution and change the scenario in time. When resistant insects are detected, fenitrothion (as wettable powder), malathion (as emulsifiable concentrate), and bendiocarb (as wettable powder) are, at the moment, the available options for *T. infestans*. Finally, it is always necessary to emphasize that house improvement and environmental management, including that of domestic animals and their shelters, are extremely relevant and needed. The great positive impact of these practices on the life standards of rural populations greatly exceeds the control of Chagas vectors. In the context of the control of triatomines, the integrated implementation of these actions with the rest of the tools available (e.g., chemical control), respecting the diversity of cultural practices of the American continent, will improve the outcomes of control programs as well as reduce the environmental impact of insecticides.

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Perspectives in Triatomine Biology Studies: "OMICS"-Based Approaches

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Abstract This chapter focuses on "omics" disciplines used in molecular entomology, in the study of triatomines, including the use of genomics, transcriptomics, metagenomics, and metabolomics. We present an initial introduction to the different methodologies including the uptake of these methods in entomology. The challenges associated with the analysis of these big datasets are discussed along with a number of commonly used tools. Subsequently, a summary of studies published to date is presented followed by a perspective for future research utilizing these technologies to answer more complex biological questions, specifically addressing triatomine biology.

Keywords Next-generation sequencing · Metagenomics · Metabolomics

1 Introduction

As in other scientific disciplines, the impact of the next-generation sequencing (NGS) technologies on molecular entomology has been impressive, taking the area of entomology to another level. In the last 15 years, mainly due to cost reduction, entomologists have successfully developed several genome and de novo transcriptome (without a reference genome) sequencing projects, which have significantly improved our knowledge of insect biology and evolution (Rinker et al. 2016; Nayduch et al. 2019). Most of these projects search for potential candidates for controlling human disease vectors and agricultural pest populations. NGS approaches provided a huge amount of information regarding the organization,

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function, and evolution of insect genomes and allowed for the identification of single-nucleotide polymorphism (SNPs) and microsatellite markers (Rinker et al. 2016; Nayduch et al. 2019). Through these projects, it has been possible to improve insect taxonomic classification (Cameron et al. 2012) while simultaneously providing a better understanding of insect populations (Neafsey et al. 2015), insecticide resistance (Weill et al. 2003), vector competence (Zhou et al. 2014), or host-seeking behavior (Matthews et al. 2018).

Following the rapid innovation of sequencing technologies and the consequential advancement in analytics, it became possible to study the metagenome (the collection of organisms associated with any given environmental sample) in a culture-independent manner. In the case of insect biology, this includes the microbial diversity associated with all bacteria, fungi, viruses, parasites, and other protozoa residing in, or on, the insect. Whether in insects or other higher organisms, it is well accepted that these organisms are important in shaping various environmental outcomes (Kong et al. 2019; Wei et al. 2017). Initial studies of microbiomes typically were focused on the conserved sequences in order to establish microbial presence and abundance (Peay et al. 2008; Pereira et al. 2010). Through NGS technologies, we are now capable of producing and handling much higher throughput at a cheaper cost, and so shotgun metagenomics, which sequences all DNA (not just selected regions), is becoming an increasingly popular alternative in ecological studies.

In the last 10 years, gene expression profiling based on NGS (RNA sequencing – RNA-Seq) has also become fundamental in molecular entomology. The possibility to create and maintain laboratory colonies under controlled conditions for many insects and the fact that researchers can easily manipulate and expose insects to different conditions and stimuli (toxins, radiation, pathogens, etc.) have both contributed to this. These factors coupled with the ability to purify huge amounts of genetic material help to explain why RNA-Seq has become so popular in the molecular entomology community. RNA-Seq allows for the characterization of expression and obtains sequences representing the entire set of transcripts from a tissue, structure, or whole body of an animal across a variety of biological conditions or from different populations (Rinker et al. 2016; Nayduch et al. 2019; Wang et al. 2009). Often, those transcripts that are differentially expressed are used in downstream behavioral and functional studies.

Understanding the genes, their expression, and their produced proteins under different conditions has been fundamental in shaping the entomology field. However, the study of metabolic products of these proteins is now also possible, thanks to the rapidly developing metabolomic field. Mass spectrometry (MS) is typically coupled with chromatography (gas or liquid) to separate samples, resulting in the identification of compound masses. MS detection sensitivities are rapidly improving, therefore allowing researchers to identify metabolites from complex biological samples with more accuracy, enabling this technique to be extensively used to complement both genetic and transcriptomic studies.

2 Omics Applications: Consideration of Technologies, Analysis Pipelines, and Outcomes for Entomological Projects

2.1 Genome Projects

Genome projects pass through different stages: (1) experimental design, (2) DNA library preparation and sequencing, (3) genome assembly, (4) structural annotation, (5) functional annotation, and (6) manual gene curation. All these stages are related and each depends on the quality of all previous steps, with the main goal of generating high-quality gene model annotations (Papanicolaou 2016; Richards and Murali 2015; Edwards and Papanicolaou 2012). The experimental design is essential and sometimes underestimated in insect genome projects. It is fundamental to select an appropriate sample (high-quality DNA), taking into consideration the genome size and the presence of possible polymorphisms and/or repetitive sequences, which are relevant aspects of high-quality assemblies (Richards and Murali 2015; Edwards and Papanicolaou 2012; Ekblom and Wolf 2014).

Second-generation technologies generate more reads, at a lower cost. However, these are shorter and directly increase the computational difficulty of genome assembly. The assembly software and algorithm(s) must be considered before choosing the sequencing strategy to obtain high-quality genome assemblies. Once Sanger and 454 technologies became obsolete, Illumina and PacBio platforms became the best options to sequence an insect genome (Papanicolaou 2016; Richards and Murali 2015; Edwards and Papanicolaou 2012). BUSCO (Simão et al. 2015) and QUAST (Gurevich et al. 2013) are excellent tools for making a quantitative evaluation of the genome assembly. The Illumina TruSeq synthetic long-read libraries and the PacBio technology allow generating long reads and are interesting alternatives to improve the quality of a genome assembly. Nevertheless, both approaches are still expensive, and in the case of PacBio, it is recommended that Illumina short reads be used to correct sequencing errors (Richards and Murali 2015).

Once a high-quality genome assembly has been obtained, the annotation process starts and is divided into the computational and gene annotation phases. For the former, data from related genomes and from species-specific transcriptomes (if available) are utilized in parallel to elaborate initial gene and transcript predictions (Ekblom and Wolf 2014). AUGUSTUS (Stanke and Waack 2003) is an ab initio gene identification tool that can be used for creating a predicted coding sequence database. Fgenesh (Salamov and Solovyev 2000), TigrScan, and GlimmerHMM (Majoros et al. 2004) can also be used in gene identification on DNA sequences. The quality of the automated predictions is usually assessed using the BUSCO software (Simão et al. 2015), which evaluates the completeness of the annotation based on the presence of orthologous genes previously identified and conserved across hundreds of genomes. The next step is to assign an identity to these predicted sequences and then find associated biologically relevant information. Usually, a local alignment tool and searches against primary (nonredundant database compiled

by the National Center for Biotechnology Information) or secondary (KO, GO, and Resistance) databases are used. This information can be complemented identifying patterns from conserved domains using tools such as Hmmer or blast searches against Pfam or CDD databases.

Although annotation tools provide acceptable results, the human touch on the second phase of the annotation is fundamental for checking and editing gene and transcript predictions. Intron leakage and gene fusion are typical errors on gene predictions that must be detected and fixed during this phase (Edwards and Papanicolaou 2012; Ekblom and Wolf 2014). Besides, core genes like odorant receptors or immune genes, which are highly polymorphic and present many paralogs, are poorly represented on the automatic predictions which in turn means that detailed and laborious searches on genome sequences are necessary (Ekblom and Wolf 2014).

In recent years, the cost reduction and easy access to NGS facilities have allowed small groups to lead and publish genome papers instead of genome consortia initiatives. However, this type of strategy usually affects the quality of the annotation and manual gene curation stages and makes data dissemination more difficult (Edwards and Papanicolaou 2012). Besides collecting, curating, and updating genomic data, insect genome projects have to start including epigenetics, population genetics, and even ecological information (Papanicolaou 2016).

2.2 Transcriptomic Studies Based on RNA-Seq

Next-generation sequencing (NGS) technologies have revolutionized transcriptome analyses through RNA sequencing (RNA-Seq), which allows for generation of mass amounts of data. However, it is a fundamental requirement to have a clear question and a deep knowledge on a given organism in order to develop a good experimental strategy that allows generation of appropriate samples and selection of sequencing conditions. Furthermore, it is important to possess know-how to accurately analyze and process sequence and expression data. The aforementioned points assist in transforming RNA-Seq and expression data into relevant biological information (Nayduch et al. 2019; Finotello and Di Camillo 2015; Conesa et al. 2016; Fang and Cui 2011).

RNA-Seq experiments are divided into three main phases: (1) sample generation and quality control, (2) sequencing, and (3) data analysis. In the first phase, the insect model, samples, and conditions should be selected together, to determine an efficient RNA extraction protocol. Researchers have to define the origin and characteristics of the samples, as well as the number of technical and biological replicates, and choose a control or basal condition (Nayduch et al. 2019; Wang et al. 2009; Conesa et al. 2016). The biological replicates are necessary to confirm that the observed results are generated by the difference between the conditions and not induced by the genetic variability across individuals. The technical replicates can be minimized since the automation of library preparation and sequencing processes

and the use of kits that tend to reduce the variation in the technical procedures; however, the minimum recommended is three per condition in order to perform a statistical analysis that allows the identification of differentially expressed genes between different treatments (Conesa et al. 2016). In some transcriptomic experiments, technical replicates are not necessary since the main goal is simply to obtain the transcript sequences to perform a detailed description of certain protein families and compare them to those of related species.

The sequencing is the second phase of an RNA-Seq experiment. Nowadays, high-throughput technologies are the most suitable for this purpose due to the reduced cost and the sequencing depth that allows revealing gene transcription (essential for the statistics) and detection of rare transcripts. Several basic parameters have to be defined according to the goal of the experiment (differential gene expression analysis and/or de novo transcript assembly) and the availability of a reference genome sequence. These parameters are read length, number of reads per sample, library type (single or paired-end), and number of replicates per treatment (Nayduch et al. 2019; Conesa et al. 2016; Korpelainen et al. 2014; Schurch et al. 2016). The Illumina read length varies between 50 and 300 base pairs (bp). Reads between 50 and 75 bp would be recommended in those cases where a well-annotated reference genome is available, such as *D. melanogaster* or *Anopheles gambiae*. In this situation, single-end libraries (only sequence from one end of the fragment is provided) would be sufficient. In other insect species, the best option is paired-end libraries (both ends of the fragment are sequenced) with a read length between 75 and 300 bp, which allows improving gene mapping and transcript reconstruction (Chang et al. 2014).

The sequencing depth (number of reads per sample) is related to the main goal of the experiments and the available data. In quantitative analysis with a well-annotated reference genome, 12–15 million raw reads per sample are satisfactory (Conesa et al. 2016; Fang and Cui 2011). In case a reference genome sequence is not available, 18–20 million reads after quality control would be suggested to obtain a good assembly and perform gene expression analysis. The read length is also important, and longer reads will provide a greater yield, increasing the coverage of the assembly. As mentioned above, the minimum recommended number of replicates is three. However, sequencing more replicates will increase the statistical power to detect smaller fold changes (Conesa et al. 2016; Fang and Cui 2011; Rapaport et al. 2013). Increasing sequencing depth could be useful to detect novel transcripts and improve genome annotation. However, if the objective is gene expression profiling, it will be more useful investing in sequencing more replicates than increasing sequencing depth (Conesa et al. 2016; Rapaport et al. 2013).

In the final phase of a transcriptomic experiment, sequencing read data have to be transformed into biological information. In an RNA-Seq experiment, two types of data can be generated from the sequencing reads: gene expression levels from selected biological material under specific conditions and transcript sequences. A general workflow of an RNA-Seq data analysis consists of the following: (1) quality control analysis of raw sequencing reads; (2) cleaning (sequencing adapters, low-quality and duplicated reads are eliminated) and trimming (bases from 5′ and 3′

ends of the read are deleted according to a threshold); (3) processed reads are mapped against a reference genome or de novo assembled and subsequently mapped against this transcriptome database; (4) in those experiments that seek to identify differentially expressed genes, the number reads that map against the genome sequences or the assembled transcriptome will be counted; and (5) gene expression analysis, in which count data are normalized (using EdgeR or DESeq packages) and after that differentially expressed genes identified. The statistical analysis will depend on the experimental design and the number of technical and biological replicates (Finotello and Di Camillo 2015; Conesa et al. 2016; Fang and Cui 2011; Korpelainen et al. 2014).

Transcript sequences can be obtained using information of read mapping against an annotated genome (genome-guided assembly), or reads can be assembled de novo. Once the transcript sequences are obtained, they can be used to identify isoforms and novel transcripts. This information can be used to improve genomic information through correction of gene models and enhance the predicted transcript database. Finally, transcript functional annotation using GO terms or KEGG pathways can be conducted (Finotello and Di Camillo 2015; Conesa et al. 2016; Fang and Cui 2011; Korpelainen et al. 2014).

At the moment, many tools and pipelines are available to process, analyze, and visualize RNA-Seq data. However, the main goal of this chapter is to provide a general view. For this reason, we suggest reading specialized publications (Finotello and Di Camillo 2015; Conesa et al. 2016; Korpelainen et al. 2014; Schurch et al. 2016; Rapaport et al. 2013) that supply deeper and detailed information about transcriptomic processing data. Thus, we will mention only a reduced list of the different processing steps of transcriptomic data together with some tools that are commonly used (Table 1).

2.3 Metagenomic Analyses

In 1990, Amann et al. proposed that approximately 99% of all microorganisms in the environment were unculturable (Amann et al. 1990). Prior to this, research surrounding the microbiota was heavily dependent on culture-based methods. Several researchers therefore began investigations into culture-independent methods for microbial analysis using genomics, eventually resulting in the field we now know as metagenomics. However, these early analyses were typically limited to bacterial sequences and based upon the conserved small subunit of ribosomal RNA (16S rRNA). The field rapidly became more sophisticated, and now these techniques are also capable of exploring eukaryotic, viral, and fungal diversity, in almost any biological or environmental sample.

Culture-independent methods for metagenomic analyses involve isolation of DNA from a target environment. This is followed by either sub-cloning into a suitable host (*Escherichia coli* is a common choice) or amplification of conserved sequences. In the former method, these clones could then be screened for specific

Table 1 Some tools used on RNA-Seq data analysis

Process	Tools	Description	Comments
Quality control analysis of sequencing reads	FastQC and NGSQC	Analyzes different parameters: base calling quality according to GC% content, % repetitive sequences, etc.	
Cleaning a trimming reads	Trimmomatic and FASTX-Toolkit	Eliminates bases with low quality from 5′ and 3′ ends; detects and eliminates Illumina adapters, duplicated reads, and those reads with low quality according to quality and minimum length thresholds	Once this step is finished, it is recommended to use quality control tools again to evaluate the remaining clean and trimmed reads
De novo assembly	Trinity[a], Oasis, Soap TransDenovo, and transABySS	Build transcripts using clean and trimmed reads	It is recommended to use tools and compare the quality of the assemblies using BUSCO and parameters like N50, number and average length of contigs and scaffolds
Read mapping	STAR, TopHat[a], and BWA	Map reads a against a reference and annotated genome	It is important to evaluate the % of mapped reads and its distribution on the genome, using, for example, IGV
	Salmon, Trinity, and Bowtie	Map reads against transcriptome assembly	It is important to evaluate the % of mapped reads
Genome-guided assembly	Trinity and Cufflinks	Uses mapping read information to build a transcriptome assembly	It can be used to identify new transcripts and isoforms and to improve gen models
Counting	HTseq, Cufflinks, RSEM, and Calisto	Generate gene/isoform count data	Different gene or isoform abundance units ((RPKM, FPKM, TPM, or counts) are used according to the sequencing and data normalization process
Differential gene expression analysis	edgeR, DESeq2, and limma	Evaluate gene/isoform expression data and use different normalization strategies and statistical analysis to identify genes differentially expressed between conditions	Understanding data manipulation and statistical analysis is fundamental for using these tools

(continued)

Table 1 (continued)

Process	Tools	Description	Comments
Data visualization	Many R packages: Gplots, ggplots2, VennDiagram, etc.	Generate different types of graphs (heat maps, volcano plots, scatter plots, etc.) using gene expression data	Very useful to visualize and detect patterns across the whole transcriptome or in specific gene sets
Functional annotation	Blast2GO and blast KEGG Orthology database	Homology-based analysis used to assign GO terms and KEGG pathways	The output is used in enrichment analysis, pathway analysis, and protein-protein interaction networks

[a]Trinity RNA-Seq de novo transcriptome assembly (github.com/trinityrnaseq/trinityrnaseq/wiki) and TopHat-Cufflinks-Cuffdiff-CummerRbund (ccb.jhu.edu/software/tophat/index.shtml) combine different tools to perform several data analysis

sequences or enzymatic pathways resulting in a more functional approach to metagenomics. On the other hand, metagenomic population analyses are typically based on amplification and sequencing of the 16S rRNA or the internal transcribed spacer (ITS) and rely on comparison to databases to find reliable matches (Baldwin et al. 1996). More recently with the advent of higher-throughput sequencing, DNA is able to be sequenced directly from the environment in a method referred to as shotgun metagenomic sequencing, removing the need for tedious sub-cloning or amplification-based protocols.

Projects based on the analysis of 16S sequences are extremely widespread among insect studies. The 16S sequence of bacterial rRNAs is highly conserved except for nine hypervariable regions that can be used to distinguish bacteria from one another. Using the entire 16S sequence is naturally more accurate; however, it is not always practical given the current read lengths involved in short-read technologies. Therefore, it is common to select two adjacent hypervariable regions for amplification with universal primers (linked to Illumina adaptors for sequencing) and subsequent analysis. Paired-end sequencing allows for both termini of a given DNA fragment to be specifically assembled and can be used to search against databases such as Greengenes (16S) (McDonald et al. 2012), SILVA (16S/18S) (Yilmaz et al. 2014), or UNITE (ITS) (Koljalg et al. 2013). One downside with this kind of analysis is that bacterial communities consist of a number of different bacteria that do not necessarily represent a clonal population of identical members. These related microbes would then contain highly similar or even identical 16S rRNA sequences. Bioinformatic pipelines have developed a way to deal with this apparent homogeneity by classifying members into operational taxonomic units (OTUs), a pragmatic solution in bacterial population genetics based on related sequences (Blaxter et al. 2005). Users are able to define the cutoff for OTU identification, with the default often set at 97% sequence identity; the same threshold for establishing different species (Stackebrandt and Goebel 1994). Once OTUs have been identified, proportions of members can be calculated, together with alpha diversity (within-sample species diversity) and the beta diversity (between-sample diversity). These

calculations give an overview of how diverse a given sample is, with respect to the microbes sequenced (Whittaker et al. 2001). Integrated pipelines exist to take sequence data all the way through to data visualization in a relatively short time (Caporaso et al. 2010; Schloss et al. 2009). Making inferences as to the specific function of a microbe in the community is difficult with just 16S rRNA sequence(s); however, tools such as PICRUSt (Langille et al. 2013) and MicrobiomeAnalyst (Dhariwal et al. 2017) have been developed in an attempt to overcome these challenges by quantifying the representativeness of genes in the community based on representative genomes. More recently, amplicon sequence variants (ASVs) have been used which group sequences based on exact sequences. This removes potential biases incorporated during amplification of marker genes and resolves sequences to the single-nucleotide level.

Alternatively, the option to sequence the entirety of a DNA sample is also possible. This formerly cost-prohibitive method is now far more accessible. One major drawback of shotgun sequencing is the analysis of such data. Analysis of these experiments is similar to genomic sequencing (Sect. 2.1), but with some modifications. One main unknown when examining metagenomes is the overall size to expect, rendering it somewhat difficult to decide on the depth of sequencing, as well as the length of reads. Longer reads using Nanopore and/or Pacbio technologies will naturally give assemblies containing longer scaffolds than those constructed with just Illumina short reads. However, the high error rate of the long-read methods generates an inherent amount of doubt in the final assemblies. Hybrid assemblies utilizing the long reads of the former technologies, together with the higher accuracy of Illumina, tend to provide the best overall results (Frank et al. 2016). One other consideration when performing assemblies is whether to assemble metagenomes on a *per*-sample basis or to co-assemble samples coming from similar environments, which may provide an overall better reference assembly but requires a read mapping step in downstream analyses to determine what is present in individual samples. Despite the difficulty associated with assembling complex microbial communities, several open-source programs have been designed specifically to address the problem (Li et al. 2015; Nurk et al. 2017; Peng et al. 2012).

Annotation of metagenomes is performed in a similar way to that of genomes. Use of common tools such as RAST (or MG-RAST, for metagenomes (Aziz et al. 2008)), Prodigal (Hyatt et al. 2010), or Prokka (Seemann 2014) is possible. However, it can be useful to incorporate other tools such as VirSorter (Roux et al. 2015) or Viral-NGS for viral annotation, as well as the tools outlined above (such as GlimmerHMM and AUGUSTUS) for annotation of eukaryotic sequences. As is the case with population metagenomics, shotgun metagenomics is complicated by the fact that it is very difficult to determine what sequences belong to which member, particularly in cases of very high relatedness. To begin to address this problem, several "binning" algorithms have been developed to sort sequences based on GC content, read coverage, tetranucleotide frequency, as well as combinations of these (Alneberg et al. 2014; Dick et al. 2009; Wu et al. 2016; Kang et al. 2015). Independently, these tools give varying results; however, DAS Tool has recently

been developed that can combine any number of different binning tools to provide consensus bins (Sieber et al. 2018).

The analysis of metagenomes is similar to that of genomes, with functional annotations playing a more prominent role. Comparisons between samples from different biological conditions can provide insight into metabolic pathways and, potentially, genetic contributions from symbionts. Databases such as eggNOG (Jensen et al. 2008), KEGG (Aoki-Kinoshita and Kanehisa 2007), or SEED (Overbeek et al. 2014) provide annotation of enzymes from orthologous clusters and thus make it significantly easier to explore metabolic pathways. In addition, several comprehensive toolboxes have been developed, such as MEGAN6 (Huson et al. 2016) and anvi'o (Eren et al. 2015), that allow users to explore their data in new ways.

Overall, the use of shotgun metagenomics in understanding the microbial ecology associated with insect biology provides an extra layer of information beyond population analyses that 16S, 18S, or ITS sequencing provides. Functional annotation of metagenomes provides a more thorough appreciation of gene content in the environment and can be easily tested for changes in gene content, which may not necessarily be reflected in changes in population dynamics, although one of the major challenges is still determining the pathways that lead to observed changes, as well as their overall functional significance.

2.4 Metabolomic Studies

High-throughput metabolomic studies can be extremely useful to resolve metabolic responses to different stimuli. It is important to note that the metabolome cannot be determined by simply using the genome and/or proteome alone. Improvements in mass spectrometry coupled with increased sensitivities of instruments have led to the development of a number of broad-spectrum, untargeted approaches to detect metabolites. These untargeted methods can identify thousands of features in individual samples. Previously, the major problem with these approaches was the annotation of features. In triatomine biology studies, this is clearly seen when researchers were forced to use the human metabolite database to annotate features, since no alternative existed (Antunes et al. 2013).

Open-source software has been a major driver of change in the metabolomics field. Analysis of the chemical spectra can be performed with tools such as MZmine2 (Pluskal et al. 2010), XCMS (Tautenhahn et al. 2012), as well as vendor-specific software. One important consideration in generating data is the sensitivity of the mass spectrometer used, since higher-resolution measurements are more likely to match to features in databases. However, related compounds can differ significantly by mass. Therefore, it is also important to generate MS fragmentation (tandem MS or MS-MS) for all major peaks. The fragmentation of compounds allows smaller structures to be matched to databases using any one of a number of available tools

(Mohimani et al. 2018; Ruttkies et al. 2016; Duhrkop et al. 2015, 2019; Wang et al. 2016).

Subsequent data analysis can be combined with other data (such as genomic, transcriptomic, and metagenomic) to map metabolic pathways with more than one datum type (Tautenhahn et al. 2012; Chong et al. 2018; Grapov et al. 2015). The challenge in these experiments is that thousands of features may be detected per sample. Compounded with in-sample differences, the absence of a one-size-fits-all extraction method and a lack of tools to analyze the data, it becomes difficult to find significant alterations in the metabolome that can be directly attributed to environmental stimuli. Nevertheless, as metabolite annotation improves and tools become freely available, this emerging field will play a more prominent role in understanding systems-level biology.

3 Metagenomics and Metabolomic Studies Associated with Triatomines

Microbiota of triatomines has been reported as early as the 1930s (Wigglesworth 1936). Since then, researchers have proposed that some insects maintain a population of obligate symbionts to supply B-complex vitamins to the insect (Baines 1956). Beyond these early studies, deeper investigations have revealed that there are more bacteria associated with the insects, albeit in varying quantities. The interest in microbiota increased with the supposition that bacteria present in the gut of insect vectors of disease may compete with parasites during colonization, including *T. cruzi* (Hurwitz et al. 2011), or that the environment is significantly altered by the presence of the parasite (Buarque et al. 2016). Overall, nine metagenomics studies have directly investigated the microbiota of triatomines. Of these, two utilized denaturing gradient gel electrophoresis (DGGE) (Gumiel et al. 2015; da Mota et al. 2012), six examined amplicons of one of the variable regions from 16S rRNA, and one developed a RADseq (Restriction-site Associated DNA sequencing) pipeline (Orantes et al. 2018). Additionally, there have been a handful of studies that have looked at the numbers of bacteria present through culturing following different stimuli (Vieira et al. 2018).

Culture-based experiments can be useful to determine relative numbers of cultivable bacteria. However, one major disadvantage in these experiments is that they fail to consider the large proportion of uncultivable species, reported to be between 42% and 99%, depending on the environment (Puspita et al. 2012). Despite this, as a part of larger studies, they have been useful in understanding changes that occur following blood feeding of triatomines, specifically effects mediated by the triatomine such as those caused by antimicrobial peptides (Vieira et al. 2014) or insect immune cells (Vieira et al. 2018; Soares et al. 2015). Studies investigating the microbiota of triatomines have also led to several proposals to use a paratransgenic approach to prevent *T. cruzi* colonization of the insects (Taracena et al. 2015).

Investigations based upon denaturing gradient gel electrophoresis (DGGE) determined that there are only a handful of bacterial species that make up the gut microbiota. Actinomycetes and *Proteobacteria* were predominant in these studies (Gumiel et al. 2015; da Mota et al. 2012), with the study results largely generated using 16S sequencing (Montoya-Porras et al. 2018). Interestingly, each of these studies appeared to have different dominant bacterial species detected, with some not detecting *Rhodococcus rhodnii* as the dominant bacterium in *R. prolixus* (Rodriguez-Ruano et al. 2018; Diaz et al. 2016), despite the fact that it was reported as an obligate symbiont in earlier research. Work by Diaz et al. was the broadest in scope, examining six different vectors challenged with *T. cruzi (*Diaz et al. 2016*)*. Their work found that *Arsenophonus* appeared as the dominant bacterium in triatomines except in *Rhodnius,* where *Pectobacterium* was instead present in higher numbers. Remarkably, examination of the salivary glands of *Triatoma* in another study also showed that *Arsenophonus* were the dominant bacteria, while in *Rhodnius* spp. *Enterobacteriaceae* (of which *Pectobacterium* is a member) tended to also be more dominant (Lima et al. 2018).

The RADseq approach collected samples of *Triatoma dimidiata* from across Central America and aimed to investigate the spread of *Trypanosoma cruzi* in wild populations (Orantes et al. 2018). The authors were able to distinguish between the vector, recent blood meal, various bacteria, and different parasite types (determined by single-nucleotide polymorphisms) present in the gut. Obtaining information such as these may ultimately help in understanding various biotic interactions occurring between insects in their environment while simultaneously collecting important strain information on parasites that they harbor.

Understanding the role of the microbiota in triatomine biology will be important in determining various other interactions that occur during metabolism. If a paratransgenic approach was to successfully be utilized to prevent *T. cruzi* colonization, it would be important to distinguish the compounds produced by the insects and those synthesized by the microbes within. One such example is *Serratia marcescens,* which reportedly produces prodigiosin, a compound that can kill the epimastigotes of *T. cruzi* Y strain (Azambuja et al. 2004). Examples such as this strongly reinforce the need for more metabolomics studies in order to properly appreciate the complex chemical ecology that occurs in the insect gut among different organisms.

One of the major challenges with high-throughput metabolomics is the lack of appropriate reference databases in order to identify molecules. Only a single study has been performed in an attempt to look at high-throughput metabolomic profiles of feces from triatomine vectors (Antunes et al. 2013). The authors noted that they were unable to identify most of the metabolites (>50%), and of those that were annotated, they were done so through comparisons to the human metabolome database. Despite these challenges, their analysis of *P. megistus*, *R. prolixus*, and *T. infestans* enabled the identification of a potential core metabolome among the three species' fecal metabolome. We expect that as analysis techniques improve, publicly available databases are continually developed, and more open-source data is generated, this field will become increasingly important in our understanding of the different biological facets associated with triatomines.

4 The *Rhodnius prolixus* Genome Project and Its Impact

According to the white paper "The case for sequencing the genome of the blood-feeding Hemipteran insect, *Rhodnius prolixus*" (vectorbase.org/sites/default/files/ftp/documents/RhodniusSeq.pdf), the idea of sequencing the *R. prolixus* genome started in November 2003 in a meeting at Montevideo (Uruguay). Two years later, the Global *R. prolixus* Genome Project Consortium was created to promote and facilitate cooperation between researchers from Brazil, Argentina, Canada, the United States, Chile, Colombia, France, Uruguay, and Paraguay. As in other genome projects, a steering committee was assembled to manage the different groups, organize meetings, and process and analyze all information. The results were published in PNAS at the end of 2015 (Mesquita et al. 2015), being the only kissing bug genome sequenced so far.

Since the publication in 2015, a total of 135 publications cited the genome paper. Most of them are focused on triatomines and include transcriptomics (Traverso et al. 2017; Marchant et al. 2016a; Brito et al. 2018; Latorre-Estivalis et al. 2017), genomics (Zumaya-Estrada et al. 2018; Martínez-Barnetche et al. 2018; Ons et al. 2016), and functional genetics studies (Wulff et al. 2017). In practical terms, the access to the genome sequences and gene annotations has allowed the researchers designing better and more accurate primers for PCR and RNA interference, facilitated heterologous expression experiments, and simplified transcriptomic and proteomic studies (it is easier and faster mapping against an annotated genome). Besides, the genomic information generated from *R. prolixus* has also been useful in other genome projects (Panfilio et al. 2019) and in transcriptomic (Lavore et al. 2018) and comparative genomic studies (Harrison et al. 2018) performed in other species.

The genome assembly and the related analyses performed during and after the publication have provided invaluable information that has been fundamental in better understanding at the molecular level, different aspects of the *R. prolixus* physiology, evolution, and vector-parasite interactions. This information together with the deep knowledge about its physiology and behavior has turned *R. prolixus* into a promising model that could be an alternative to *D. melanogaster, Bombyx mori*, or *Apis mellifera* to study relevant aspects of insect biology.

5 Transcriptomic Studies in Triatomines

A total of 26 transcriptomic studies in triatomines have been published in the last 15 years (Table 2). *Rhodnius prolixus* and *Triatoma infestans* are the most studied species within triatomines, with nine and eight publications, respectively. For other triatomine species, the number of transcriptomic studies is reduced, with four publications for *Triatoma pallidipennis* and *Triatoma brasiliensis* and three for *Triatoma*

Table 2 Description of the transcriptomic studies performed in triatomines

Species	Methodology	Tissue	Main results	References
Rhodnius prolixus	Cloning step followed by sequencing on Perkin Elmer 9700 (Beckman Coulter, Inc.)	Salivary glands	The generation of a sialotranscriptome database	Ribeiro et al. (2004)
	Cloning step followed by sequencing on ABI 377 equipment (Applied Biosystems)	Midgut and fat body in response to bacterial (*E. coli* and *Micrococcus luteus*) and *Trypanosoma cruzi* infections	Identification of immune-related molecules from the fat body and intestine	Ursic-Bedoya and Lowenberger (2007b)
	Cloning step followed by sequencing on ABI 377 equipment (Applied Biosystems)	Different stages of ovarian follicle development	Transcripts with potential roles in oogenesis and embryo development were characterized	Medeiros et al. (2011)
	454-Roche	Segments of the digestive tract (anterior midgut, posterior midgut, and rectum) from adult females before feeding, 12 h, 24 h, 2 days, and 5 days after blood meal. Whole body, fat body, ovary, testes, and Malpighian tubule were also sequenced	Identification of several families of enzymes associated with the digestion, immunity, signal transduction, amino acid metabolism, and detoxification	Ribeiro et al. (2014)
	454-Roche and Illumina HiSeq	Antennae, rostrum, and head from male and female adults	Compared different assembly strategies (de novo and based on a reference genome) and proposed reference-based assembly after genome annotation as the most appropriated	Marchant et al. (2016a)

(continued)

Table 2 (continued)

Species	Methodology	Tissue	Main results	References
	Illumina HiSeq	Antennae from fifth-instar larvae and female and male adults	The antennae showed increased expression of several chemosensory-related genes in imaginal bugs, while both sexes had similar expression patterns for most target genes	Latorre-Estivalis et al. (2017)
	454-Roche and Illumina HiSeq	Central nervous systems from unfed,1-h, 4-h, and 24-h post-blood-fed insects	Description of sequences and expression profiles from enzymatic superfamilies that are thought to mediate xenobiotic detoxification and resistance	Traverso et al. (2017)
			Identification of G protein-coupled receptors for opsins and neurohormones	Ons et al. (2016)
	Illumina HiSeq	Ovaries	Identification of central components of the piRNA pathway	Brito et al. (2018)
Rhodnius montenegrensis and *Rhodnius robustus*	Illumina HiSeq	Head and salivary gland from adults	They found a high degree of genetic divergence between the two species and likely corroborate the species status of *R. montenegrensis*	De Carvalho et al. (2017)
Triatoma infestans	454-Roche	Embryos and diverse organs (reproductive and digestive tract, Malpighian tubules, brain, fat body, and salivary glands) of fed and starved insects of the five nymphal stages, adult mated females and adult males	Description of neuropeptide precursor genes	Traverso (2016)

(continued)

Table 2 (continued)

Species	Methodology	Tissue	Main results	References
			Description of the humoral and cellular innate immune components	Martínez-Barnetche et al. (2018)
			Description of sequences from enzymatic superfamilies that are thought to mediate xenobiotic detoxification and resistance	Traverso et al. (2017)
			They identified putative immune-related homologs	Zumaya-Estrada et al. (2018)
	454-Roche	Nymph integument	Identify which genes are expressed and their putative role	Calderón-Fernández et al. (2017)
	Illumina MiSeq	Salivary glands	The generation of a sialotranscriptome database	Schwarz et al. (2014)
	Cloning step followed by sequencing on CEQ 2000 DNA instrument (Beckman Coulter)		The generation of a sialotranscriptome database	Assumpção et al. (2008)
	Cloning step followed by pyrosequencing (no details on manuscript)		The generation of a sialotranscriptome database	Kato et al. (2010)
Triatoma dimidiata	454-Roche	Embryos and diverse organs (reproductive and digestive tract, Malpighian tubules, brain, fat body, and salivary glands) of fed and starved insects of the five nymphal stages, adult mated females and adult males	Description of neuropeptide precursor genes	Traverso (2016)

(continued)

Table 2 (continued)

Species	Methodology	Tissue	Main results	References
			Description of the humoral and cellular innate immune components	Martínez-Barnetche et al. (2018)
			Description of sequences from enzymatic superfamilies that are thought to mediate xenobiotic detoxification and resistance	Traverso et al. (2017)
			They identified putative immune-related homologs	Zumaya-Estrada et al. (2018)
Triatoma pallidipennis	454-Roche	Embryos and diverse organs (reproductive and digestive tract, Malpighian tubules, brain, fat body, and salivary glands) of fed and starved insects of the five nymphal stages, adult mated females and adult males	Description of neuropeptide precursor genes	Traverso (2016)
			Description of the humoral and cellular innate immune components	Martínez-Barnetche et al. (2018)
			Description of sequences from enzymatic superfamilies that are thought to mediate xenobiotic detoxification and resistance	Traverso et al. (2017)
			They identified putative immune-related homologs	Zumaya-Estrada et al. (2018)
Triatoma brasiliensis	454-Roche Illumina Hiseq	Antennae and rostrum	The performance of ten assembly workflows was compared	Marchant et al. (2015)

(continued)

Table 2 (continued)

Species	Methodology	Tissue	Main results	References
	Illumina HiSeq	Antennae and rostrum from sylvatic and domiciliary individuals from both adult sexes	They found under-expressed sensory transcripts in the domiciliary bugs compared to the sylvatic and in females compared to males	Marchant et al. (2016b)
	Illumina MiSeq	Nymph heads	They reported a transcriptome assembly and annotation	Gonçalves et al. (2017)
	Cloning step followed by sequencing on Mega-BACETM1000 instrument (GE/Amersham Biosciences)	Salivary glands	The generation of a sialotranscriptome database	Santos et al. (2007)
Triatoma rubida	Cloning step followed by sequencing on CEQ 2000 DNA instrument (Beckman Coulter)	Salivary glands	The generation of a sialotranscriptome database	Ribeiro et al. (2012)
Triatoma matogrossensis	Cloning step followed by sequencing on CEQ 2000 DNA instrument (Beckman Coulter)	Salivary glands	The generation of a sialotranscriptome database	Assumpçao et al. (2012)
Panstrongylus megistus	Illumina HiSeq	Salivary glands	The generation of a sialotranscriptome database	Ribeiro et al. (2015)
Panstrongylus lignarius	Illumina HiSeq	Salivary glands and fat body	The generation of a sialotranscriptome database and characterization of transcripts expressed in fat body	Nevoa et al. (2018)

(continued)

Table 2 (continued)

Species	Methodology	Tissue	Main results	References
Dipetalogaster maxima	Cloning step followed by sequencing on CEQ 2000 DNA instrument (Beckman Coulter)	Salivary glands	The generation of a sialotranscriptome database	Assumpção et al. (2011)

dimidiata. For the remaining kissing bugs (there are more than 140 species), only 1 study was performed (more details are displayed in Table 2).

The first transcriptomic study was performed in 2004 by Ribeiro and collaborators, and they characterized the sialotranscriptome (the set of transcripts expressed in the salivary glands) of *R. prolixus* by means of Sanger technology. Since then, Ribeiro and collaborators have characterized the sialotranscriptome of other triatomines (nine species, including *R. prolixus*), observing differences in the amount and type of proteins (mainly on lipocalins, serine proteases, and antigen 5-like proteins) expressed in the salivary glands. The sequencing of the transcripts produced by this tissue together with those from the head was used to clarify the taxonomic relationship between *Rhodnius montenegrensis* and *Rhodnius robustus* (De Carvalho et al. 2017).

The enzymatic superfamilies that mediate xenobiotic detoxification and insecticide resistance (Traverso et al. 2017), humoral and cellular innate immune components (Martínez-Barnetche et al. 2018), neuropeptide precursor genes (Traverso 2016), and putative immune-related homologs (Zumaya-Estrada et al. 2018) were characterized by generating using normalized libraries using different organs from diverse developmental stages (embryos, fifth-instar nymphs, and adults from both sexes) and physiological conditions (unfed and fed) of *T. infestans*, *T. pallidipennis*, and *T. dimidiata* (Table 2). The transcripts expressed in the antennae of *R. prolixus* (Latorre-Estivalis et al. 2017) and in the antennae and rostrum of *T. brasiliensis* (Marchant et al. 2016b) have been characterized. These transcriptomic studies have allowed identifying sensory proteins (including odorant receptors, odorant binding and chemosensory proteins) that are essential for the detection of pheromones and environmental stimuli related to host-seeking behavior.

The fat body and different segments of the digestive tract were also characterized at the transcriptomic level (Ursic-Bedoya and Lowenberger 2007a; Ribeiro et al. 2014; Nevoa et al. 2018). Interestingly, Ursic-Bedoya and Lowenberger (Ursic-Bedoya and Lowenberger 2007a) carried out the only transcriptomic study that analyzes the effects of *Trypanosoma cruzi* infection in a Chagas disease vector so far. Ribeiro et al. (Ribeiro et al. 2014) conducted an exhaustive description of the transcript production along the digestive track at different times after blood ingestion. This study provided a better understanding of the mechanisms involved in blood digestion in *R. prolixus* and the vector-parasite interaction. However, no quantitative analysis was performed due to the sequencing technology (Roche-454). The effect

of blood ingestion was also analyzed for the central nervous system by Ons et al. (Ons et al. 2016). Regarding other tissues, the central components of the piRNA pathway (Brito et al. 2018) and transcripts with potential roles in oogenesis and embryonic development (Medeiros et al. 2011) were described through transcriptomic experiments of *R. prolixus* ovaries. Finally, transcriptomic data from *R. prolixus* and *Triatoma brasiliensis* were used to evaluate the efficiency of different transcript assembly workflows (Marchant et al. 2015, 2016a).

6 Perspectives

6.1 Comparative Genomics

Rhodnius prolixus is the only triatomine whose genome has been sequenced so far (Mesquita et al. 2015). The authors showed that *R. prolixus* presents differences in the immune signaling pathways and gene expansions related to chemoreception, feeding, and digestion processes that have enabled adaptation to a blood-feeding lifestyle. Sequencing other triatomine genomes, especially those from relevant vectors like *T. brasiliensis* or *T. infestans*, would allow performing comparative analysis between kissing bugs and other insects. In addition, this would confirm whether these distinctive genomic features are exclusive to *R. prolixus* or characteristic traits of triatomines. Furthermore, differences regarding vector competence, geographical distribution, and the capacity for ecological adaptation among triatomine species could be analyzed from a genomic perspective.

Genomic and transcriptomic sequences can be used to identify new molecular markers (such as single-nucleotide polymorphisms: SNPs) that could further improve the classification of triatomines, which was already extended based on morphological traits and/or ribosomal and mitochondrial markers (Bargues et al. 2010; Mas-Coma and Bargues 2009). These methods would help to clarify the relationship between *R. prolixus* and *Rhodnius robustus*; the structure and composition of *T. phyllosoma*, *T. brasiliensis*, and *T. infestans* complexes; or the cryptic species that belong to the *Triatoma dimidiata* complex (Bargues et al. 2010; Mas-Coma and Bargues 2009).

6.2 Hybridization and Introgression Events

Introgression (incorporation of genes from one set of differentiated populations into another) and hybridization (crossbreeding of different species and the generation of completely fertile offspring) have been observed in laboratory colonies (*R. prolixus* and *R. robustus* are virtually indistinguishable morphologically) and in several wild populations, such as the *T. infestans* complex (Mas-Coma and Bargues 2009),

compromising their identification. Thus, genome and transcriptome data from triatomine hybrids will provide a new perspective of these microevolutionary phenomena in insects at the molecular level and may identify new molecular markers. It would be especially relevant in investigating cases such as that of *T. infestans*-resistant populations from the Gran Chaco region (Stevens and Dorn 2017).

One other aspect related to the hybridization of insect species that should be considered is that as climate change proceeds, the environment for triatomines will expand. Indeed, triatomine species are reported worldwide including Asia (Liu et al. 2017) and Europe (Schmunis and Yadon 2010). This expansion raises new environmental challenges for triatomines as monitoring the gene flow in hybrid populations may reveal insights into niche adaptation by the insects.

6.3 Population Dynamics and Vector Control

As in other insects, microsatellite, mitochondrial, and ribosomal sequences have been widely used to study population genetics in the Triatominae (Mas-Coma and Bargues 2009; Stevens and Dorn 2017) providing fundamental information about ecological and evolutionary processes (Stevens and Dorn 2017). RNA-Seq data comparing insects from different populations will provide genetic information that would complement that generated by classical markers. This approach could be used to clarify the geographical and ancestral origin of *T. infestans* that represent Andean (Bolivia and Peru) and non-Andean populations (Stevens and Dorn 2017).

Besides clarifying the origin or composition of a certain population or species, the transcriptomic characterization of different triatomine populations will be useful to understand two aspects that are fundamental in Chagas disease epidemiology: the occupation of sylvatic and domestic/peridomestic ecotopes and the genetic bases of insecticide resistance. Both factors determine the human-vector interaction and the effectiveness of Chagas disease control campaigns. Regarding the first point, *T. dimidiata* will be an excellent model since it has been captured in sylvatic and domestic/peridomestic ecotopes across different countries of Central and South America. However, its distribution among ecotopes is not uniform (e.g., populations are located in domestic or peridomestic habitats in Southern Guatemala, while populations are sylvatic in the North) (Stevens and Dorn 2017). Transcriptomic characterization of different *T. dimidiata* populations could estimate the genetic flow between domestic and sylvatic populations and whether adaptation to different ecotopes, especially human-inhabited areas, relies on molecular elements. This type of comparison may also be performed at the interspecies level comparing, for example, data from populations of *T. dimidiata* and *T. infestans* coming from domestic ecologies. Interestingly, molecular changes have already been reported for domestic populations of *T. brasiliensis,* which presented a reduced expression of various odorant binding and chemosensory proteins compared to sylvatic insects (Marchant et al. 2016b) as well as in other insects such as *Aedes aegypti*, which presents a "domestic" form with an increased expression and ligand sensitivity of an odorant

receptor (*AaegOr4*) involved in the recognition of a compound from human odor (McBride et al. 2014). Obtaining this kind of data for triatomines would help to understand the domiciliation process of several populations and provide new avenues in developing vector control strategies.

In triatomine control campaigns based on insecticide treatment, determining insecticide effects on the genetic variation of a population, the source of dwelling reinfestation and monitoring insecticide resistance are key points (Stevens and Dorn 2017). In this sense, the effect of insecticide campaigns has been studied in *T. infestans* populations from Argentina and Bolivia in the Gran Chaco region using microsatellites and mitochondrial-ribosomal markers (Stevens and Dorn 2017). The transcriptomic characterization of Gran Chaco populations, e.g., before and after insecticide applications, in insects from sylvatic vs. insecticide-treated areas or insect populations with different levels of insecticide resistance, will provide information regarding changes induced by insecticide application on triatomine populations. In particular, this will help to determine the origin of surviving insects (domestic/peridomestic or sylvatic ecotopes) and their molecular traits (presence of specific mutations and/or altered expression of detoxification enzyme-coding genes (Grosso et al. 2016)) and the potential genetic flow between the different areas and ecotopes. This information will help to increase the effectiveness of Chagas disease vector control programs.

Several mutations in the active site of pyrethroids (knockdown resistance – *kdr*) have been described in *T. infestans* populations from the Gran Chaco region of Argentina and Bolivia (Sierra et al. 2016; Capriotti et al. 2014; Fabro et al. 2012). The presence of these mutations has been associated with different levels of insecticide resistance in the region (Sierra et al. 2016). The transcriptomic characterization of these populations together with toxicological and biochemical tests, such as the determination of the median lethal dose (LD50), will be very useful to understand triatomine insecticide resistance phenomena at the genomic, transcriptomic, and metabolic levels. This information will be fundamental to identify new molecular markers that allow detecting the emergence of insecticide-resistant individuals (or populations) in its early stages and guide vector control strategies to avoid the establishment of resistant populations in inhabited areas.

6.4 Insecticide Resistance in Laboratory Colonies

The study of insecticide resistance using kissing bugs from laboratory colonies with different phenotypes and under different experimental and physiological conditions is also an interesting approach to understand this phenomenon. Availability of large numbers of insects coupled with an ease to maintain some kissing bug species under laboratory conditions facilitates these experiments. The transcriptomic responses after insecticide administration could be characterized in different tissues, such as the central nervous system, target sites of many common insecticides such as the fat body where many xenobiotics are metabolized and degraded, or the integument that

forms the first barrier to insecticides and hinders their entrance into the organism. Studies such as this could be performed at different time points following insecticide administration, using different compounds and doses, and comparing the responses between sensitive and resistant phenotypes. This information will provide a global view of the metabolic processes triggered by insecticides on insects.

6.5 Molecular Basis of Triatomine Behavior

Kissing bug behavior has been studied with the aim to control their populations since they are insect vectors of Chagas disease. In addition, some of them are excellent models to study neuroethological processes in insects due the existing knowledge on their physiology and behavior. Since the publication of the *R. prolixus* genome, it has been possible to explore the molecular aspects of triatomine behavior in depth.

As in other human disease vectors, the sensory system is fundamental in host-seeking behavior since it allows triatomines to detect several cues emitted by hosts, such as odors (including CO_2), water vapor, and infrared radiation (Barrozo et al. 2016). Sexual, flight, oviposition, and aggregation-related behaviors in triatomines also rely on their sensory system (Barrozo et al. 2016). The chemical blends that constitute sexual and aggregation pheromones from different triatomines have been characterized (Pontes et al. 2008; Vitta et al. 2009), as well as relevant host-emitted odorants (Guerenstein and Guerin 2001). In the last years, receptors involved in different sensory modalities were annotated in the *R. prolixus* genome (Mesquita et al. 2015); and RT-PCR (Zermoglio et al. 2015; Latorre-Estivalis et al. 2016), RT-qPCR (Latorre-Estivalis et al. 2015), and RNA-Seq (Latorre-Estivalis et al. 2017) experiments were used to describe the expression patterns and transcriptomic changes of some of these receptors. In addition, expression of several sensory receptors and proteins of *Triatoma brasiliensis* was described by Marchant and Mougel (Marchant et al. 2016b). Nevertheless, few odorant receptors have been characterized at a functional level (Franco et al. 2015, 2018), predominantly due to the large number of receptors identified in the *R. prolixus* genome (116 odorant receptors and 33 ionotropic receptors) but also due to the absence of genomic and transcriptomic information from other triatomines, rendering it difficult to select specific candidates to perform functional genetics studies. Once genomic and transcriptomic data from other triatomine species become available, the comparison of their sensory protein repertoires, especially OR, IR, OBP, and CSP families which tend to change at higher rates, will be relevant to understand adaptation and speciation processes in kissing bugs, as with other insects (Neafsey et al. 2015; Gardiner et al. 2008; Rinker et al. 2013a). A positive selection analysis of the identified orthologs may reveal receptors that potentially act on the detection of relevant environmental stimuli, like host odors or pheromone components, which are shared between kissing bug species.

Transcriptomic studies of sensory organs (antennae, tarsi, and rostrum) from insects in different physiological and developmental conditions would be an excellent strategy to identify key receptors mediating plastic triatomine behaviors. In this sense, the transcriptomic characterization of the antennae (considered the main sensory organ of insects) from starved and fed insects, on different days after ecdysis or in different hours during the day, will be informative about genes involved in triatomine foraging, as observed for mosquitoes (Rinker et al. 2013b; Tallon et al. 2019; Rund et al. 2013). In parallel, transcriptomic changes at central level could be characterized (generated through RNA-Seq experiments using the brains from the same group of insects) to analyze the relation between the central nervous system and the periphery in the control of insect behavior.

RNA-Seq experiments could also be used to identify receptors (Bengtsson et al. 2012) mediating sexual and oviposition behaviors, by comparison of transcriptomic data from virgin female and male adult antennae, as it was already performed in moths (Bengtsson et al. 2012). Additionally, the transcriptomic characterization of other sensory organs, like the tarsi, rostrum, and genitalia from kissing bug adults, will be relevant. Indeed, the expression of several ORs and IRs has already been reported for these tissues in *R. prolixus* using RT-PCR *(*Latorre-Estivalis et al. 2016*)*. However, no functional information is available so far. Contact chemoreceptors from tarsi are involved in the detection of sweet, bitter, and saline substances in *Apis mellifera* (de Brito Sanchez et al. 2014), sexual pheromone in *D. melanogaster* (Inoshita et al. 2011), and DEET in *Aedes aegypti* (Dennis et al. 2019). A deep characterization of the receptor's genes transcribed in these structures, together with electrophysiological experiments, would be useful to understand the role of tarsi in triatomine sensory physiology. Regarding the genitalia, comparing RNA-Seq data from female and male adults may identify receptors potentially mediating mating and oviposition in triatomine adults. All transcriptomic information related to the sensory function and its regulation could be used to manipulate triatomine behavior through the development of better ligand-based baits (Duvall et al. 2019) or in silico design of receptor-blocking agents (Boyle et al. 2013; Kepchia et al. 2019).

6.6 Exploitation of Sequencing Technologies for New Insights into Biological Adaptations

Above we have outlined several strategies that would help better understand the mechanistic pathways that lead to particular behaviors or physiological changes. As NGS technology has evolved, so have sample preparation strategies that can provide more detailed insights into particular mechanisms. Therefore, exploitation of these new sample preparation approaches will yield further details regarding specific pathways. One area of research that has yet to be examined in triatomines is epigenetics. With PacBio sequencing, methylation patterns on the DNA of insects are readily accessible. The potential epigenetic effects – those that are passed to new

offspring – have not been investigated in triatomines. DNA methylation is important in proper regulation of the genome, and it has been linked to social activities as well as behavioral features of different insect species (Yan et al. 2014). If this regulatory mechanism is related to behavioral traits is totally unexplored but may provide mechanistic insights into how these behaviors are passed on to new progeny.

On the other hand, investigations of insect transcriptomes could be significantly enhanced by using modified sample preparation methods. For example, IsoSeq, developed as a method for identifying new isoforms of RNAs on the PacBio platform, would also be useful in identifying transcript isoforms in triatomines with respect to various behaviors. An updated bioinformatic pipeline has made this even more accessible, yielding greater isoform diversity which may help to resolve the regulatory structure of complex networks or multigene families (Sahlin et al. 2018). Another technique (among many others) is TimeLapse-Seq, which uses a convertible nucleoside intermediate to identify newly produced transcripts (Schofield et al. 2018). The major advantage of this technique over conventional RNA-Seq is that, given an experimental condition, one can distinguish transcripts that are produced immediately following a condition from those that were present prior to the start of the experiment, thus narrowing down the specific pool of RNAs to compare. Ultimately, this approach generates a much more accurate dataset for comparative transcriptomic analyses. As these and other methods develop further, their use in triatomine research will provide significantly deeper insights into the molecular mechanisms behind behavioral, morphological, and other changes.

6.7 Triatomine-Trypanosome Interaction

As in other vector infectious disease systems, the relationship between triatomines and trypanosomes has been extensively studied over different approaches and methodologies (see review in (Guarneri and Lorenzo 2017)). Despite this, several fundamental aspects of triatomine biology remain unresolved. As it was shown in the previous sections, few "omic" studies have been performed on triatomines in the context of trypanosome infection, particularly with respect to the microbiome. Therefore, the study of the triatomine-trypanosome relation through "omic" disciplines presents significant potential, especially as more genomic and transcriptomic data for triatomines and trypanosomes are generated.

Trypanosoma cruzi differentiates and multiplies as it passes through the triatomine digestive tract. At the same time, the parasite has to navigate the immune response and other physiological processes triggered by triatomine blood digestion (Guarneri and Lorenzo 2017). The events activated in the insect during the life cycle of the parasite could be analyzed using genetic, metagenomic, and metabolomics approaches across several tissues, at different time points and in different developmental stages following infection with different parasite strains. The effects should then be evaluated in triatomines presenting an acute or a chronic parasite infection.

With respect to the *T. cruzi* life cycle in the insect gastrointestinal tract, the first critical event occurs in the anterior midgut (AM), where a severe reduction in the incoming trypomastigote population has been reported within the first 24 h (Ferreira et al. 2016). However, the exact mechanisms leading to this reduction are totally unexplored. This topic should be approached from two separate angles: the triatomine response could be investigated through the temporal characterization of the AM transcriptomic response originating following the ingestion of an uninfected or infected blood meal and the microbial response, including that of the parasite, using techniques such as shotgun metatranscriptomics. The surviving trypomastigotes reach the posterior midgut (PM) approximately 1 day after blood ingestion where they differentiate into epimastigotes. This form adheres to the perimicrovillar membrane and multiplies. Several molecules produced by this membrane that are critical to this interaction have already been identified (Guarneri and Lorenzo 2017), yet as above, the precise response of the microbiota in this section of the gut has not been characterized. Finally, the parasites reach the rectum where they become metacyclic trypomastigotes, the infective form of parasite. The adhesion to the rectal wall seems to be critical in this differentiation as well as the nutritional status and the presence of a number of molecules produced in the rectum (Atwood et al. 2005).

Paratransgenic control of Chagas disease vectors was first attempted in the 1990s (Durvasula et al. 1997; Beard et al. 1992). These studies relied on known symbionts, which were determined through culture-based experiments. However, more recent techniques have uncovered a number of new candidates for paratransgenic control of Chagas disease (Diaz et al. 2016). In fact, one such candidate, *Serratia marcescens,* produces prodigiosin, a compound found to inhibit the growth of *T. cruzi* Y strain (Azambuja et al. 2004). This highlights the importance that the microbiome plays in the regulation of these parasites. Therefore, a much deeper understanding of the metabolites and the bacteria, fungi, and viruses in the gastrointestinal tract of triatomines will be an essential point of future research and may also yield some insight into niche adaptation in sylvatic and domestic environments.

The immune response triggered by the presence of *T. cruzi* could be investigated by means of RNA-Seq experiments of AM, PM, and rectum, again generated from healthy and infected triatomines. In parallel, the study of the proteomes from epimastigote and metacyclic trypomastigote forms (Jr et al. 2005) as well as those from the PM and rectum from infected insects would be an interesting approach to identify new proteins involved in the *T. cruzi*-triatomine interaction. Similar approaches have already been performed in other host-parasite systems (Cuesta-Astroz et al. 2019). This approach could be used to analyze potential differences on vector competence presented among Chagas disease vectors, by mining of proteomes from different parasite strains and those corresponding to the digestive tract regions in several infected vectors. Furthermore, coupled with more genome sequences of triatomines, it would be possible to analyze the dynamics between the genetics of *T. cruzi* and their vectors. This kind of comparative analysis could be extended to include the proteome, considering the ability of parasites to infect and colonize the insect gut. This approach would benefit from experimental infection and may yield

some insight into the evolutionary adaptations that have led to a vector being able to carry, and transmit, any given strain of parasite.

It has been shown that the fitness of different triatomine species is affected following *T. cruzi* infection. For example, a reduction in the life span, the number of eggs laid, molt delay, or other behavioral alterations have been described in infected insects. The harmful effects depend on the parasite strain, insect species, environmental conditions, and nutritional status (Guarneri and Lorenzo 2017). However, the physiological processes and molecular events underlying these alterations have been poorly studied (Marliére et al. 2015). Transcriptomic profiling experiments from the central nervous system and ovaries generated from healthy and infected triatomines may reveal significant information regarding the molecular mechanisms involved in behavioral and reproductive alterations caused by the parasite. A better understanding of potential behavioral manipulation processes promoted by the parasite is crucial, since vector-human contact could potentially be modified, increasing the risk of disease transmission.

Interestingly, another trypanosome species, *Trypanosoma rangeli,* is known to infect triatomines. However, it is not pathogenic to humans, presents a different life cycle within the insect with a migration to the salivary glands where the infective forms appear (only in *Rhodnius* spp.), and promotes pathogenic effects on their insect hosts (Guarneri and Lorenzo 2017). These closely related trypanosome species are an excellent system to study the coevolution of trypanosomes and their vectors since both are capable of infecting the same insect species while presenting extensive differences in their respective life cycles within the vector, in addition to the differences in pathogenesis resulting in varying levels of impact on insect fitness. In this sense, the study of RNA-Seq experiments and predicted proteomes from the parasites and the different insect tissues would allow identifying specific genetic changes and protein-protein interactions that will be very informative to study the parasite-vector coevolution.

6.8 Ecdysis in Hemimetabolous Insects

Most of the studies related to ecdysis have been performed using holometabolous insects as models. Triatomines represent an excellent model to study the ecdysis of hemimetabolous insects since the events (pre-ecdysis, ecdysis, and post-ecdysis), their intervals, and associated behaviors are already well described (Ampleford and Steel 1982). Several studies performed in *R. prolixus* have described the roles of different molecules, mostly neuropeptides and hormones, during the different phases of ecdysis using RT-qPCR and RNAi techniques (Wulff et al. 2017; Lee et al. 2013). However, no study has analyzed ecdysis in hemimetabolous insects from a global perspective at both the transcriptomic and metabolomic levels. For this reason, temporal transcriptomic and metabolomic characterizations of central nervous system extracts after blood ingestion would reveal fundamental information to understand the complex cascade of events involving nuclear receptors,

peptide hormones, neuropeptides, and their related receptors that are involved in the control of *R. prolixus* ecdysis and provide a first more general insight into the ecdysis of hemimetabolous insects.

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