Toxinology

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Anita Malhotra *Editor*

Evolution of Venomous Animals and Their Toxins

Springer

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Editor-in-Chief
P. Gopalakrishnakone

In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and micro organisms in detail to understand their mode of action on targets. On the other hand, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their three interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology. Composed of 12 volumes, *Toxinology* provides comprehensive and authoritative coverage of the main areas in toxinology, from fundamental concepts to new developments and applications in the field. Each volume comprises a focused and carefully chosen collection of contributions from leading names in the subject.

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Editor-in-Chief

Anita Malhotra
Editor

Evolution of Venomous Animals and Their Toxins

With 89 Figures and 10 Tables

Springer

Editor-in-Chief
P. Gopalakrishnakone
Venom and Toxin Research Programme
Department of Anatomy
Yong Loo Lin School of Medicine
National University of Singapore
Singapore, Singapore

Editor
Anita Malhotra
School of Biological Sciences
Bangor University
Bangor, Gwynedd, UK

ISBN 978-94-007-6457-6 ISBN 978-94-007-6458-3 (eBook)
ISBN 978-94-007-6459-0 (print and electronic bundle)
DOI 10.1007/978-94-007-6458-3

Library of Congress Control Number: 2016962206

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Science+Business Media B.V.
The registered company address is: Van Godewijckstraat 30, 3311 GX Dordrecht, The Netherlands

Series Preface

The term TOXIN is derived from the Greek word *Toeikov* and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 12 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

P. Gopalakrishnakone
M.B.B.S., Ph.D., F.A.M.S., D.Sc.
Editor-in-Chief

Acknowledgments

I would like to sincerely thank the section editor of this volume, Anita Malhotra, for the invaluable contribution of her expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Sarah Mathews, Sunali Mull, Meghna Singh, Mokshika Gaur, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

P. Gopalakrishnakone
M.B.B.S., Ph.D., F.A.M.S., D.Sc.
Editor-in-Chief

Volume Preface

Thomas Dobzhansky famously wrote "*Nothing in Biology Makes Sense Except in the Light of Evolution*" in his 1937 essay, a statement that firmly established evolution as a unifying concept in biology. To put it in another way, while classical toxinology investigated the "what" and "how" questions about animal toxins, the "why" questions involving the wider evolutionary context in which venomous animals developed was often overlooked. However, I am glad to say that this is no longer the case, and recent years have seen a free exchange of ideas between toxinologists and evolutionary biologists, with benefits to each field. Thus, this volume opens with a section containing chapters on the wider evolutionary context of venom in animals, the molecular evolutionary processes involved in generating diversity, and the concept of venom evolution as being driven by an arms race that also involves evolution of resistance to toxins by prey.

As an evolutionary biologist, I am also strongly convinced that we can only study the evolution of toxins if it is underpinned by a clear understanding of the evolution of the animals that they evolved within. The original concept of the book was that a chapter on the evolution of venom and toxins within a particular group of venomous animals (Sect. 2) would be balanced by a chapter giving the latest understanding of the systematics of that group (Sect. 4). Our understanding of relationships changes all the time as new data or new methods of analysis become available, and the *Handbook of Toxinology* format is eminently suited to frequent updating.

Finally, the volume finishes with a section of the evolution of venom delivery systems. The definition of a venomous animal, as opposed to a poisonous one, encompasses the evolution not just of toxins but also a specialized mechanism for administering them by injection. While venom delivery systems have been well studied in some groups, such as snakes, we are only just beginning to learn about the origins and amplification of these systems in other groups, such as centipedes.

I am deeply grateful to the many authors who contributed to this volume for agreeing to share their expertise. I also wish to express my thanks to Professor Ponnampalam Gopalakrishnakone for inviting me to edit this volume and to the professional and patient editorial team at Springer (particularly Audrey Wong, Sunali Mull, and Sarah Mathews) for their support during the process.

October 2016 Anita Malhotra
Bangor, Gwynedd, UK B.A. (Oxon), Ph.D., F.L.S, F.R.S.B., F.Z.S.L

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Editor-in-Chief

P. Gopalakrishnakone
Venom and Toxin Research Programme
Department of Anatomy
Yong Loo Lin School of Medicine
National University of Singapore
Singapore, Singapore

P. Gopalakrishnakone, M.B.B.S., Ph.D., F.A.M.S., D.Sc., is professor of anatomy and chairman of the Venom and Toxin Research Programme at Yong Loo Lin School of Medicine, National University of Singapore, where he has become an emeritus professor.

Prof. Gopal has also got a new appointment in the newest University in Singapore, Singapore Institute of Technology (SIT), as a professor of anatomy in the Health and Social Sciences Cluster. Prof. Gopalakrishnakone is also a consultant to the Defence Science Organization in Singapore; adjunct senior research scientist at the Defence Medical Research Institute; and an honorary principal fellow at the Australian Venom Research Unit, University of Melbourne, Australia.

His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employs include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of Toxicon, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000

Award in Singapore; and the Research Excellence Award from the Faculty of Medicine at NUS (2003).

His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Professor Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

About the Editor

Anita Malhotra
School of Biological Sciences
Bangor University
Bangor, Gwynedd, UK

Anita Malhotra received her B.A. in Zoology from Oxford University (Jesus College) in 1985 and Ph.D. from the University of Aberdeen in 1992. Dr. Malhotra then moved to Bangor University in 1994, where she took up a lectureship in 1995. She is now director of the Graduate School of the College of Natural Sciences. She is a molecular ecologist and evolutionary geneticist, who developed the first robust and largely comprehensive molecular phylogenies for Asian pitvipers, based on both mitochondrial and nuclear markers. This work revealed a number of cryptic species and resulted in the radical revision of the taxonomy of the group. Dr. Malhotra has used this phylogeny to test hypotheses about the evolution of specific venom components such as the phospholipase A2 enzymes, using a multidisciplinary genomic, proteomic, and functional approach. She has active collaborations all over the world with other academic institutions and research-based SMEs (particularly in Europe, India, China, and Japan). Other research involves investigation of the dynamics of natural selection, vicariance and evolutionary response to changing environmental conditions, and honeybee genetics in the UK. Author of over 100 publications, her work regularly attracts media attention and been covered by BBC Wildlife, the Sunday Telegraph, and Radio 4 as well as in local news media in the UK and abroad. Dr. Malhotra was a recipient of the Zoological Society of London's Thomas Henry Huxley Award and Marsh Prize in 1992 and a coauthor on the article that received the 2004 Joseph B. Slowinski Award for Excellence in Venomous Snake Systematics. She is joint coordinator for East Asia on the IUCN Viper Specialist Group and is also a guest professor at Shenyang University and Yibin University in China.

Contributors

A. L. Allcock School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

Kevin Arbuckle Department of Ecology, Evolution and Behaviour, University of Liverpool, Liverpool, UK

David Baracchi Research Centre for Psychology, School of Biological and Chemical Sciences, Queen Mary University of London, London, UK

Nikeisha Caruana Department of Ecology, Environment and Evolution, La Trobe University, Melbourne, VIC, Australia

Nicholas R. Casewell Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool, UK

Ira R. Cooke Department of Biochemistry and Genetics, La Trobe Institute for Molecular Sciences, La Trobe University, Melbourne, VIC, Australia

Life Sciences Computation Centre, Victorian Life Sciences Computation Initiative, Melbourne, VIC, Australia

Allen M. Cooper Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA, USA

Marymegan Daly Museum of Biological Diversity, The Ohio State University, Columbus, OH, USA

Michel M. Dugon Discipline of Zoology – School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

Sebastien Dutertre Institut des Biomolécules Max Mousseron (IBMM) – UMR 5247- Equipe Sciences Analytiques des Biomolécules et Modélisation Moléculaire, Université de Montpellier 2, Bat. 17, Montpellier, France

Alexander E. Fedosov A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia

Bin Gao Group of Peptide Biology and Evolution, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Adam D. Hargreaves Department of Zoology, University of Oxford, Oxford, UK

William K. Hayes Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA, USA

Yuri I. Kantor A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia

Harald M. I. Kerkkamp Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Leiden, The Netherlands

Richard James Lewis Centre for Pain Research, Institute for Molecular Bioscience, The University of Queensland, St. Lucia/Brisbane, QLD, Australia

Rodrigo Ligabue-Braun Graduate Program in Cellular and Molecular Biology, Center for Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

Stephen P. Mackessy School of Biological Sciences, University of Northern Colorado, Greeley, CO, USA

Anita Malhotra School of Biological Sciences, Bangor University, Bangor, Gwynedd, UK

Gillian M. Mapstone Department of Life Sciences, The Natural History Museum, London, UK

Thomas M. McCabe School of Biological Sciences, University of Northern Colorado, Greeley, CO, USA

Mrinalini Department of Biological Sciences, National University of Singapore, Singapore, Singapore

John F. Mulley School of Biological Sciences, Bangor University, Bangor, Gwynedd, UK

David R. Nelsen Department of Biology and Allied Health, Southern Adventist University, Collegedale, TN, USA

Mark Norman Sciences, Museum Victoria, Carlton, VIC, Australia

Jutty Rajan Prashanth Centre for Pain Research, Institute for Molecular Bioscience, The University of Queensland, St. Lucia/Brisbane, QLD, Australia

Nicolas Puillandre Institut de Systématique, Evolution, Biodiversité ISYEB – UMR7205 – CNRS, MNHN, UPMC, EPHE, Muséum National d'Histoire Naturelle, Sorbonne Universités, Paris, France

Torsten H. Struck Department of Research and Collections, Natural History Museum, Oslo, Norway

Jan M. Strugnell Department of Ecology, Environment and Evolution, La Trobe University, Melbourne, VIC, Australia

Simon Tragust Animal Population Ecology, Animal Ecology I, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, Bayreuth, Germany

Abigail S. Tucker Department of Craniofacial Development and Stem Cell Biology, King's College London, Guy's Hospital, London, UK

Freek J. Vonk Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Leiden, The Netherlands

Naturalis Biodiversity Center, Leiden, The Netherlands

Molecular Ecology and Evolution Group, School of Biological Sciences, Bangor University, Bangor, UK

John H. Werren Department of Biology, University of Rochester, Rochester, NY, USA

Brooke Whitelaw Department of Ecology, Environment and Evolution, La Trobe University, Melbourne, VIC, Australia

Jeremy J. Wright New York State Museum, Albany, NY, USA

Shangfei Zhang Group of Peptide Biology and Evolution, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Shunyi Zhu Group of Peptide Biology and Evolution, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Part I

The Wider Evolutionary Context

Evolutionary Context of Venom in Animals

1

Kevin Arbuckle

Abstract

Much of the research on venoms has understandably focused on clinical implications of human envenomation and detailed molecular studies of toxins. However, as with any biological trait, venom exists in an evolutionary context and must be considered as such if we are to gain a full understanding of the biology of animal venoms. Consequently, this chapter aims to provide an overview of the diversity of venom and venomous animals and also a set of evolutionary principles which are particularly applicable here. There has been substantial variation in the definition of "venom" and "venomous" in the literature, so this is discussed first with the aim of giving a definition which encompasses a number of important features of venoms. A survey of the functional diversity of venoms and taxonomic diversity of venomous animals is then provided as an introduction to the evolutionary drivers of venom and how it is distributed across the animal tree of life. The last three sections consider three principles that are important to venom evolution: (1) the composition of venom is variable both between and within species; (2) venom evolves in the context of antagonistic coevolutionary interactions; and (3) venom can have consequences for the ecology and evolution of animals that possess it beyond its direct functions to their behavioral ecology.

Keywords

Diversity • Ecology • Function • Terminology • Variation

K. Arbuckle (✉)
Department of Ecology, Evolution and Behaviour, University of Liverpool, Liverpool, UK
e-mail: k.arbuckle@liverpool.ac.uk

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_16

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Introduction

Venomous animals have a long history of inspiring fear, fascination, interest, and dread in humans – perhaps since the earliest evolution of our species. They have also been the subject of a great deal of scientific study over the last several hundred years. The majority of this research has focused on two areas. Firstly, and largely because humans are understandably self-obsessed, the medical implications of envenomation are intensely studied, including epidemiology, toxicological effects, and clinical treatment. Secondly, because venoms typically consist of a diverse set of individual toxins (Casewell et al. 2013) with a wide range of specific and often potent physiological actions (Fry 2005), much research on molecular aspects of venom toxins has been undertaken, including structure-function relationships, toxin evolution, and "venomics" (e.g., proteomic and transcriptomic studies of venom). In fact, these two areas of venom research have been so prolific as to form the major content

of several recent books, including Chippaux (2006), Mackessy (2009), Fry (2015), and the current *Handbook of Toxinology* series.

However, despite the impressive advances that clinical and molecular toxinological studies have achieved, much less attention has been paid to the broadscale evolutionary context of animal venoms. The ecological relations of whole venoms are often considered little more than a sidenote to detailed investigation of particular toxins, but these are necessary to understand the selection pressures and therefore drivers of venom evolution. Therefore, this chapter aims to provide a review of the diversity of, and the principles underlying the evolution of, venoms.

What is Venom?

Before a meaningful discussion of the evolution of venomous animals can be made, it is important that the terminology is made clear. Therefore, this section begins with a broad overview of different weapons in the chemical arsenal of animals in order to set the context within which venom falls, and then the definition of venom (and therefore venomous) is clarified.

The Diversity of Chemical Warfare in Animals

Collectively, animals use a wide range of chemicals for several purposes (see "Venom Functions" section) but primarily for defense against predators. Venom is only one form of chemical defense (or offense), but it is useful to consider the additional forms that it can take because many of these fulfill the same function from the ecologically relevant perspective of predators. This subsection refers to chemical defense specifically because, with the notable exception of venom, all other traits considered here are used in defensive roles as opposed to other functions. The unique characteristic of venom, that it can be deliberately and directly transferred into the body of another organism, is likely to be an important factor in the ability to use venom for a broader range of functions.

When considering antipredator mechanisms, it is useful to think about them in the context of Endler's (1986) five stages of predation: detection, identification, approach, subjugation, and consumption. In principle, defenses can operate to disrupt predation attempts at any one of those stages. However in practice, with one exception, chemical defenses typically act only at the later stages of predation (against subjugation and consumption of prey), perhaps because such defenses are effective but carry high energetic costs (e.g., Higginson et al. 2011) and so are reserved for late-stage "emergency" situations. Indeed, many chemically defended animals use other strategies such as protective coloration (e.g., aposematic warning signals or camouflage) to avoid attack at earlier stages of predation. Furthermore, the exception to late-stage chemical defenses is using a low nutrient composition of body tissues to discourage predation (Bullard and Hay 2002): a rare and unusual

strategy that carries constraints such as an ability to store substantial energy reserves and does not involve energetically costly ways to biosynthesize or sequester chemicals.

Avoidance of subjugation by predators can use several forms of chemical defense. Firstly, slime or mucous production can either make the prey "slippery" and so difficult to subdue or adhere to the predator's jaws (which restricts their movement), depending on the properties of the mucus. Secondly, venom is often used as a defense against subjugation by causing pain and/or actual harm to predators during attack. Thirdly, a somewhat more cryptic form of chemical defense is the production of detoxifying chemicals, including antibodies, as one way to confer resistance against the venom of predators. Fourthly, there are various chemicals which are sprayed toward predators and have various effects that act from a distance (i.e., before the prey is fully subjugated). Some of these spray defenses are well known and include many iconic species, such as the high-temperature bombardier beetle spray, horned lizards that squirt blood from behind their eye, and spitting cobras that spray venom with contact toxicity to eyes.

Once predators have subdued prey, limited options exist to avoid consumption, and these can generally be considered to be the release of chemicals that are ingested by predators, hopefully before the prey animal is mortally injured. The effects of such chemical defenses can be either (or a combination of) simple distastefulness, irritation to the mucous membranes of the predator's mouth (such as the blistering caused by some millipede secretions, pers. obs.), nausea (inducing vomiting or other sickness but not "harmful" per se), or poisoning (causing physiological harm to the consumer). Poisons are the most intensively studied of these, and along with venoms are the most studied chemical defenses more generally. However, it is remarkably difficult to distinguish these effects in many cases, and so for many such defenses we do not know, for example, whether a predator rejects prey under natural conditions because it does not like the taste, experienced some discomfort (from irritation or sickness), or was fully poisoned.

The different "anti-consumption" defenses highlighted in the last paragraph are often discussed collectively as "poisons," though this is an overly general classification. Even more broadly, the word "poison" is sometimes used erroneously to include venoms, but these are important to distinguish for medical, evolutionary, ecological, or behavioral studies. Therefore, following the focus of this chapter on venoms, the following subsection attempts to provide a meaningful definition of "venom."

In Search of a Definition

Although the questions of "what is venom" and therefore "what constitutes a venomous animal" may seem obvious to many, a unanimously accepted definition of "venom" has been surprisingly difficult to develop. This is perhaps also the reason that much literature, even recent work, has continued to describe venom as "salivary

secretions" and other such terms that limit the connectivity and cohesiveness of the field (e.g., Hildebrandt and Lemke 2011). A recent review by Nelsen et al. (2014a) highlighted the many different definitions that exist in the literature and makes a good attempt at summarizing these. In essence, much of the disagreement exists due to the emphasis placed on different aspects of venoms. For instance, the relative importance of the potential to inflict damage on humans may be considered an important defining characteristic by medics but less so (or not at all) by those from molecular genetic, biochemical, evolutionary, or ecological backgrounds.

Medical importance of envenomation to humans, although a useful distinction for clinical and public health purposes, is of little relevance to a biologically meaningful definition of venom. If we accept that all of biology occurs in the context of evolution, including venoms, then a meaningful definition of venom must include evolution as a main component. Because humans have not been important in the origin of venoms in any species, any such interactions with humans are demonstrably misguided as a part of the definition. This is highlighted in the many species that use venom effectively to catch their prey but whose venom is of little or no clinical importance for humans. Beyond this, purely objective arguments for what is, and is not, important for defining "venom" are lacking. Nevertheless, we can consider those factors that are most commonly used in the literature to generate a definition that is sensible, meaningful, and consistent with as much of the existing literature as possible.

Nelsen et al. (2014a) found that the following attributes are frequently used in definitions of venom: (1) the venom is produced or stored in a gland, (2) there is a specialized delivery system used to transfer the venom to another organism, (3) the venom is transferred via an injury, (4) the venom is actively (as opposed to passively) transferred to another organism, and (5) the venom functions in predation and/or defense. In addition, Fry et al. (2009a) also add another characteristic that is commonly used, implicitly or explicitly, in definitions of venom: (6) the venom contains molecules ("toxins") which interfere with physiological or biochemical processes in another organism. These six defining characteristics of venom are generally sensible, but there are two points that do not capture the range of organisms traditionally considered venomous. Firstly, attribute 1 would not consider cnidarians (such as jellyfish) to be venomous as they do not possess a venom "gland" per se, but they do possess a sub-glandular apparatus that fulfills the same function (specialized "nematocyst" cells). Therefore, a better attribute would be that the venom is produced and/or stored in a specialized structure, which may include both glands and nematocysts. Secondly, attribute 5 would not consider the platypus to be venomous because they do not use venom (at least primarily) in either predation or defense but in intraspecific competition. Therefore, this attribute should be relaxed to include other potential venom functions, such that the venom need only function to provide a benefit to the venomous animal once it is transferred to another organism, albeit this is most commonly a predatory or defensive benefit.

A definition of venom can therefore be ascribed based on the six attributes above (with the modifications discussed) alongside the recognition that evolution is

important and medical consequences to humans are not important for this purpose. To this end, the following should provide a definition that represents a synthesis of current use and key components of venom:

> *Venom is a biological substance produced by an organism that contains molecules* ("*toxins*") *which interfere with physiological or biochemical processes in another organism*, *which has evolved in the venomous organism to provide a benefit to itself once introduced to the other organism. The venom is produced and/or stored in a specialized structure and actively transferred to another organism through an injury by means of a specialized delivery system*.

Venom Functions

As briefly touched on in the preceding section, venoms are often considered to function in predation or defense (e.g., Edstrom 1992), with some literature giving additional mention to the use of venom in competition (e.g., Casewell et al. 2013; Fry et al. 2015). However, the true diversity of venom functions is broad but somewhat masked by such short and simple statements. This is not surprising because venom, as defined above, should provide many different potential benefits, and the evolution of diverse animal ecologies opens the door for a wide range of venom functions. Consequently, this section will highlight the diversity of venom functions found in animals as this directly relates to the selection pressures driving the evolution of venoms.

One key point to bear in mind is that the following functions are not mutually exclusive: venom may be used for different functions by the same animal, although not necessarily equally (one function may still be the main evolutionary driver). For instance, the evolution of snake venoms is primarily driven by their use in predation (Barlow et al. 2009; Daltry et al. 1996), but that does not prevent their use as a very effective antipredator defense often with devastating consequences for the person or other animal bitten (e.g., Boyer et al. 2015; Chippaux 2006; Mackessy 2009). In addition to this example of co-opting venom for another purpose than its main driver, some animals have specifically evolved a "dual-purpose" venom systems which include separate predatory and defensive components, such as cone snails (Dutertre et al. 2014) and scorpions (Inceoglu et al. 2003).

Predation

A predatory function for venom is arguably the most common primary driver of venom evolution (see phylogenetic distribution of venom functions in Casewell et al. 2013). It is also the main function of venom in many of the better known lineages of venomous animals such as snakes, spiders, and scorpions. Nevertheless, there are various ways in which venoms can be used to aid predation.

The most direct is also the most obvious – incapacitating prey to allow the venomous predator to consume it. Note that the function here is to incapacitate

prey, not necessarily to kill it. Killing prey would usually require more venom than incapacitation, which is unlikely to be favored by evolution since venom is energetically expensive to produce (McCue 2006; Morgenstern and King 2013) and incapacitated prey is just as beneficial as killed prey for consumption. That is not to say that killing prey as a standard predatory tactic is not common, in fact many mechanisms for incapacitation may kill with more time, but that killing is not an essential part of predatory venom functions. This is reflected in the toxicological effects of many predatory venoms, which typically cause two main classes of symptoms in prey: interfering with nerve action causing paralysis (and often death later) and altering blood and blood vessels causing blood loss and associated shock. For example, ant-specialist spiders (*Zodarion*) are capable of predating prey much larger than themselves by using paralyzing venom (Pekár et al. 2014), without the need to actually kill the prey before consumption. Furthermore, Fry et al. (2009b) demonstrated that the Komodo monitor (*Varanus komodoensis*) inflicts a deep wound with recurved, serrated teeth and uses its venom to quickly induce loss of consciousness in prey via the onset of shock. The same authors also suggested that the extinct *Varanus priscus* probably used a similar strategy.

Venom has also been considered to aid in predation after prey consumption by increasing the speed of digestion with proteolytic toxins. This is most commonly discussed in snakes because viper venoms often have relatively high proteolytic activities and because some of the earlier studies demonstrating an effect of venom on digestion were carried out using *Crotalus atrox* rattlesnakes (Thomas and Pough 1979). Recent studies, such as McCue (2007), have failed to find an effect on digestion in *Crotalus atrox*, which has been used to call into question whether increased digestive performance can be a function of venom. However, McCue (2007) conducted experiments at higher temperatures (25–30 °C) that did not produce a large effect in Thomas and Pough (1979) – the latter authors found that venom was more important in digestion at lower temperatures (15 °C). This, combined with some evidence of improved digestive efficiency conferred by the venom of some other species, such as *Andrallus spinidens* bugs (Zibaee et al. 2012), suggests that such a function cannot be completely discounted in studied taxa and is certainly a plausible function that has not been well studied in most venomous animals.

Finally, parasitism represents another predatory (in the broad sense) function of venoms in some animals. Blood-feeding parasites such as ticks and vampire bats often produce anticoagulant venoms that facilitate prolonged feeding by maintaining a constant flow of blood, alongside other actions (Cabezas-Cruz and Valdés 2014; Low et al. 2013). In addition, parasitoids present an interesting situation wherein the predation is not by the organism that injected the venom but by its offspring at a later date. In *Asobara* parasitoid wasps, the venom acts to paralyze the host during egg laying by the wasp before killing it at a later date (Moreau et al. 2009) – ensuring a stationary and storable food source for the larvae when they hatch. More detailed transcriptomic work on the parasitoid wasp *Nasonia vitripennis* has revealed that the venom of this species induces a variety of changes to gene expression in the host (Martinson et al. 2014). The venom of this wasp causes the host to enter

developmental arrest and also upregulates certain antimicrobial peptides that likely help to prevent spoilage of the (live but immobile) host until the larvae hatch out and consume the host.

Defense

Aside from predation, defense is the most common primary function for venoms, especially antipredator defense. Furthermore, as alluded to earlier in the context of venomous snakes envenomating potential predators, many (probably most) venomous species will use their venom in a defensive role even if the main role is, for example, predation. However, several groups of animals, such as bees and sea urchins, use venom primarily for defense, and many others (such as spitting cobras) regularly use venom for both defense and another function. In spite of this variation in functional importance, there are some generalities that can be made for defensive venoms when compared to predatory or other venoms.

Firstly, defensive venoms tend to be simpler in composition than predatory venoms (Casewell et al. 2013), likely because the latter are involved in more intense arms races which generates selection for diverse and fast-evolving venoms (see later section on "Antagonistic Coevolutionary Interactions Are the Common Thread in Venom Evolution"). Secondly, defensive venoms are more likely to have evolved to be effective at a distance, such as spitting cobras or spraying behavior of certain scorpions (Nisani and Hayes 2015). This enables the venomous animal to defend itself while keeping away from the predator but requires chemical components which can penetrate external surfaces (mostly eyes or mucous membranes). Thirdly, defensive venoms are likely to contain toxins which interfere with fast-acting physiological processes such as nerve transmission – because lengthy delay in actions can give the predator enough time to kill the animal before the venom takes effect. Consequently, many defensive venoms contain toxins which act to cause paralysis quickly by blocking neuromuscular receptors or to target pain receptors to cause instant and intense pain (Bohlen et al. 2011; Dutertre et al. 2014; Inceoglu et al. 2003; Siemens et al. 2006).

Although antipredator defense is particularly well studied (and probably more important), some venoms are also known or suspected to contribute toward immune and antiparasite defense. For instance, in some social hymenopterans, the venom is spread over the cuticle of other individuals and the nest combs, and appears to reduce the prevalence of infections via antimicrobial venom components (Baracchi et al. 2011). In others, they actively apply venom to fungus-infected group-mates which helps eliminate the fungus (Tragust et al. 2013). Similarly, Grow et al. (2015) have shown that the venom of slow loris species (*Nycticebus*) is effective in killing arthropods that are similar to those which parasitize them, and that lorises anoint themselves with the venom. However, it is difficult to know to what extent the venom is transferred to the microbes or parasites per se, and therefore it is debatable whether such uses would be considered as those of venom (even if the same secretions function as a typical venom in

other circumstances). The alternative is to consider the same substance both as a venom and as a contact poison, depending on the use at any one time.

Intraspecific Competition

Few venoms seem to have a prominent function in intraspecific competitive interactions, but this is known in a few mammal species, namely the platypus (*Ornithorhynchus anatinus*) and slow loris species (*Nycticebus*). It is notable that both of these groups also use their venom in defense and possibly other functions but that they nevertheless use venom to a large extent for competition. Platypus venom glands increase in size during the breeding season in males, and scars from envenomations are usually found in males, both of which highlight the predominance of intrasexual competition for females as a driving force in the evolution of their venom (Whittington et al. 2009). Echidnas (*Tachyglossus* and *Zaglossus*) were once thought to also be venomous and indeed possess similar glands to the platypus, but their "venom" system is highly degenerate and the secretions now seem to function in scent communication during the breeding season rather than as venom (Wong et al. 2013). Slow loris venom appears to also be used in intraspecific competition based on the frequency, patterns, and consequences of bite wounds on wild lorises as well as observations in captivity (Nekaris et al. 2013), though this is less well studied than in the platypus.

Reproduction

A potentially unique venom function is found in scorpions of the genus *Hadogenes*. These species are extremely reluctant to use venom in either predation (relying on their pedipalps) or defense, but during courtship males will sting females in the side, which seems to produce sedative and perhaps aphrodisiac effects (Leeming 2003). Other scorpions have occasionally been seen stinging during courtship, but similar behavioral responses are not observed, and *Hadogenes* also possess marked sexual dimorphism wherein males have much longer tails which facilitates this behavior. Therefore, *Hadogenes* represents an interesting genus for studies of sexual selection (including sexual dimorphism of venom apparatus), toxin evolution, and potentially a source of new pharmaceutical drugs given that the unique function may be associated with unique toxins. However, the venom of the genus has been extremely understudied and almost nothing is known about the details.

Taxonomic Distribution of Venomous Animals

Venom is a trait which has evolved multiple times across the animal tree of life (Fig. 1), a testament to the myriad benefits it confers and corresponding selection pressure to originate and maintain venom systems across diverse groups of animals (Fig. 2).

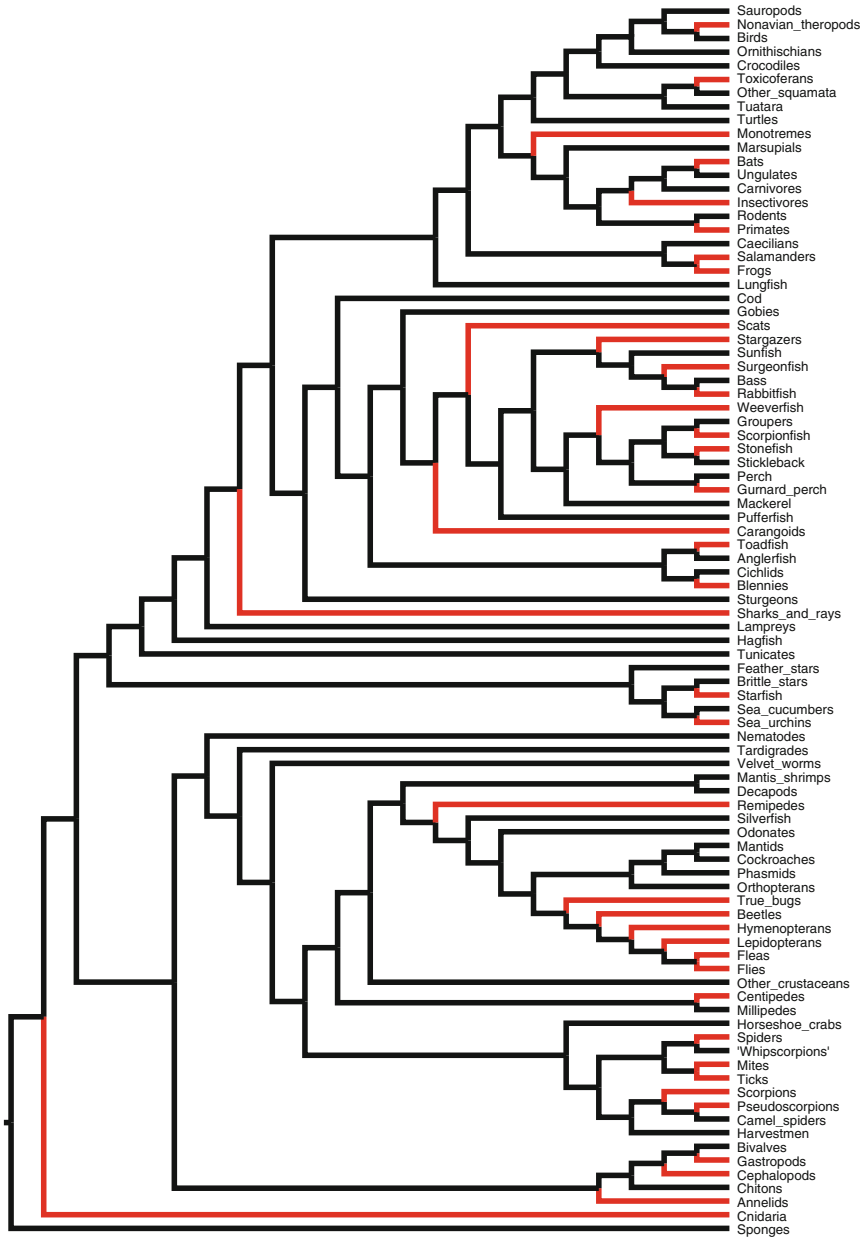

Fig. 1 Phylogenetic tree of animals highlighting groups containing venomous species in *red*. Taxa were selected solely to show the diversity of venomous animals, not to give an overview of animal diversity in general. Topology is based on the Tree of Life Web Project (www.tolweb.org) in addition to many studies from the literature which were used to resolve uncertainty in the ToL project phylogeny

Fig. 2 Examples of venomous animals. From left to right in each row: young huntsman spider (*Heteropoda* sp.), blue leg centipede (*Scolopendra mirabilis*), adder (*Vipera berus*) (row 1); unidentified squid from Thailand, flat rock scorpion (*Hadogenes troglodytes*) (row 2); nursery web spider (*Pisaura mirabilis*), unidentified tick from Uganda, assassin bug nymph (*Rhynocoris* sp.) (row 3); stony coral (*Favia* sp.), cuttlefish (*Sepia officinalis*), unidentified wasp from Uganda (row 4); leaf-cutter ant (*Atta cephalotes*), beadlet anemone (*Actinia* sp.), Komodo monitor (*Varanus komodoensis*) (row 5). (All photos by the author)

It should be noted that Fig. 1 is a substantial underestimate of the number of times venom has evolved in animals as it only represents broad taxonomic groups, within which venom may have evolved more than once. It is also likely that future research will uncover more groups of venomous animals because many groups are understudied and research effort is directed toward medically relevant species rather than an objective survey of animal life.

Although the focus of this chapter is on venomous animals, it is noteworthy that venom is not restricted to animals. Other groups of organisms more commonly use other forms of chemical defenses, but venom is used by taxa as diverse as plants and bacteria. For instance, plants in the family Urticaceae have stinging hairs which pierce the skin of mammals and inject pain-inducing venom which deters herbivores (Iwamoto et al. 2014). In the case of the stinging tree (*Dendrocnide* spp.), the pain has reported to be excruciating and has caused deaths of domestic animals and humans (Hurley 2000). One species of bacteria, *Photorhabdus luminescens*, has so far been reported to have a toxin delivery system which would qualify it as truly venomous, although the diversity of bacteria and the lack of detailed study of most mean that it is likely that more examples will be revealed in the future. In *P. luminescens*, a "syringe protein" penetrates the cells of an insect host and transfers a toxin molecule through the pore of the "syringe" into the insect cells (Gatsogiannis et al. 2013).

Cnidarians

All groups of cnidarians (including jellyfish, corals, and sea anemones) use venom in either predation or defense (Fig. 2). This is produced in specialized cellular structures, called nematocysts, and is injected into prey or predators using a harpoon-like venom apparatus that is extremely sensitive and readily fired upon contact (Fautin 2009). The venom contains a number of neurotoxins which can cause pain and rapid death from respiratory paralysis (Edstrom 1992) – the latter mostly in prey, but some species are capable of delivering lethal envenomations to humans and other predators. Of additional interest is the ability of some specialist predators of cnidarians, especially nudibranch mollusks, to extract nematocytes intact from eaten cnidarians (without discharging them), transport them to their own skin surface, and employ the "stolen" venom system in their own defense (Greenwood 2009).

Annelids

Two of the three major groups of annelid worms possess venom – the exception is oligochaete worms (such as earthworms) which are not known to have any venomous species. On the other hand, blood-feeding leeches possess venom with similar functional characteristics to many other parasitic animals. Specifically, the venom contains toxins which prevent blood clotting and therefore maintain sufficient flow to enable prolonged feeding and also suppress inflammation, pain, and other immune responses in the host to prevent detection of the leech and removal before feeding is complete (Hildebrandt and Lemke 2011).

The third major group of annelids, polychaete worms, have evolved a range of venom systems and so presumably have also evolved venom multiple times. Among the best studied of these are bloodworms *Glycera* spp., which feed on other invertebrates and inject a neurotoxic venom with their jaws which quickly immobilizes prey and can be used to give a painful bite in defense (Edstrom 1992). Other polychaetes, such as the various species known as "scale worms," are considered to have a venomous bite but are very poorly known (von Reumont et al. 2014a). In addition to species which inject venom using mouthparts, other polychaetes (e.g., Amphinomida or bristle worms) have evolved a venom apparatus consisting of fragile spines (actually modified chaetae) which function defensively by breaking off when touched, causing a wound into which venom is delivered (von Reumont et al. 2014a). This group of worms feeds on slow-moving or sessile prey, and this, combined with the position and mechanism of venom delivery, restricts the benefits of this venom to antipredator defense.

Mollusks

Venom in mollusks is largely restricted to two of the major groups: gastropods and cephalopods (Fig. 2). However, as mentioned above in the subsection on "Cnidarians," nudibranch mollusks can reallocate the nematocysts of their cnidarian prey to their own surface and use it in defense. This is a highly unusual form of sequestration of chemical defenses, which usually entails the extraction of particular toxins or toxin precursors from the diet but in this case involves the sequestration of the entire venom apparatus.

Many gastropods (snails) contain venom which is used primarily to aid predation and injected via a barbed "tooth" (radula) which is thrust into prey. It is also used in defense, and some species of cone snail (*Conus* spp.) are capable of lethal envenomation of humans and other predators. Although many groups of marine gastropods are known to possess venoms (Edstrom 1992; von Reumont et al. 2014a), that of cone snails is by far the best studied and has generated a vast quantity of literature. *Conus* spp. venom contains a number of highly potent neurotoxins which serve to rapidly incapacitate (by respiratory paralysis) fast-moving prey such as fish, which would otherwise be unavailable to a slow-moving predator such as the snails. However, *Conus* have a dual-purpose venom system which produces separate venoms for predation and defense and so has presumably faced strong selection pressure for both functions (Dutertre et al. 2014). It has recently been suggested that in this genus venom originally evolved in worm-eating species for defense against predatory fish, and this subsequently allowed the shift to hunting mollusks and fish using the venom for predation (Dutertre et al. 2014), although how or if this also applies to other venomous gastropods is uncertain.

Cephalopods have long been known to possess venom, but recently it has been revealed that this is likely to be far more widespread than previously thought, evolving early in the group (Fry et al. 2009c). Although also used in defense, the venom primarily functions in predation with toxins causing paralysis in prey

organisms such as crabs (Cornet et al. 2014). There may also be some digestive function of the venom of some species, including *Octopus vulgaris*, although this is based on more circumstantial evidence (Edstrom 1992). Blue-ringed octopuses (*Hapalochlaena*) are famed for being one of the few cephalopods capable of killing a human, and their venom contains tetrodotoxin, among other components. The tetrodotoxin is contained both in the venom and within the body as a defensive poison. In addition, female blue-ringed octopuses actively provision their offspring with tetrodotoxin to ensure they have adequate defense early in life (Williams et al. 2011).

Centipedes

The venom system of centipedes features a unique means of injecting venom – the first pair of walking legs evolved modifications over 400 mya to be used as venom-delivering pincers (Dugon and Arthur 2012) (Fig. 2). This means that, in essence, centipedes do not bite but instead have a venomous pinch. The venom itself is used as an effective defense, but its evolution has likely been primarily driven by its role in predation (Yang et al. 2012). The venom contains a complex mixture of toxins with diverse effects, but the overarching results from a pinch occur in two phases (Undheim and King 2011). Firstly, fast-acting but short-lived toxins produce pain and hypotension (as well as some muscle paralysis) almost immediately, which contributes to prey being quickly driven into shock. Secondly, slower-acting toxins produce a pain which spreads from the pinch site, some breakdown of the skeletal muscle, and cardiac arrest.

Crustaceans

Perhaps surprisingly, given the high diversity of crustaceans (even considered as a paraphyletic group excluding insects), only one single species has (very recently) been found to have venom. Von Reumont et al. (2014b) uncovered venom consisting of a paralyzing neurotoxin and a large number of proteinase and other enzymes from venom glands of *Xibalbanus tulumensis* – a cave-dwelling remipede crustacean. Almost nothing else is known about this venom so far, although it is plausible that the venom has evolved to enable efficient predation in an environment highly conducive to prey escaping if not quickly subdued. If this is the case, then venom may be genuinely rare in crustaceans for as yet unknown reasons, but von Reumont et al. (2014a) have expressed an expectation that more venomous crustaceans will be found with further study, especially in parasitic groups.

Arachnids

Spiders and scorpions are among the first animals that come to mind when thinking about venomous creatures (Fig. 2). However, the literature on the venom toxinology has been slower to amass than their cultural impact. Furthermore, at least three other

groups of arachnids also possess venom: mites, ticks (Fig. 2), and pseudoscorpions. A very limited amount of evidence exists to suggest that camel spiders (or solifugids) may also be venomous (von Reumont et al. 2014a). This would explain the reportedly high levels of pain from their bites, but currently enough information to confidently consider them venomous is lacking.

Recent investigations have provided much insight into the composition of spider venoms – though interpretations and reviews have often been strongly focused on medical importance to humans (e.g., Sannaningaiah et al. 2014). Nevertheless, the primary function of all spider venoms is undoubtedly predation. Consequently, it can enable spiders to subdue and eat much larger prey than they would otherwise (Pekár et al. 2014). Although spiders are well known for biting defensively, only ~10–20 of the 50,000 species of spider have a venom capable of causing serious consequences to human predators, and even infamous species such as the widow spiders (*Latrodectus*) are remarkably reluctant to bite in defense (Nelsen et al. 2014b). These observations further suggest that spiders primarily use venom to catch prey rather than in defense, and the venom often contains specific insecticidal and paralyzing neurotoxins (Sannaningaiah et al. 2014).

Scorpion venom is not as clearly streamlined into a single function as spider venom. As highlighted above (in the "Venom Functions" section), scorpions have a dual-function venom incorporating separate antipredator defense and predatory components. Scorpions have one type of venom (sometimes called prevenom) which is expelled initially and differs in appearance from the subsequently expelled venom – in *Parabuthus transvaalicus* the prevenom is clear, whereas the secondary venom is milky white (Inceoglu et al. 2003). The first venom type is particularly adapted to instill immediate and severe pain in mammals as an antipredator deterrent, whereas the second venom type is better adapted for causing paralysis and death in their insect prey (Inceoglu et al. 2003). This is likely to be at least in part due to the action of different toxins which act specifically on either mammals or insects (Ochola et al. 2007). Additionally, one genus of scorpions (*Hadogenes*, flat rock scorpions) appears to have co-opted its venom primarily for use in courtship, a potentially unique venom function (see "Reproduction" subheading in "Venom Functions" section).

All ticks and some mites are blood-feeding parasites, and consequently they use a venom rich in toxins that interfere with blood to ensure a steady flow during feeding (Andersen 2010). Ticks are known to have venom components that reduce the immune response by the host to avoid detection while feeding (Cabezas-Cruz and Valdés 2014). Furthermore, both ticks and mites can induce paralysis in hosts via their venom, which again likely acts to prevent the host from removing the parasite before it is finished feeding (Cabezas-Cruz and Valdés 2014; Tomalski et al. 1988). Therefore, in common with other blood-feeding parasites, ticks and parasitic mites contain venoms which function almost exclusively in promoting this type of feeding ecology.

Pseudoscorpions represent the most understudied arachnids that are known to be venomous. Their venom system is integrated into their pedipalps, which have a "venom tooth" at the ends which connects to a duct from the venom gland. As a

consequence of the lack of research, there is currently little known about the venom of pseudoscorpions, but it seems to function in predation and may be a key factor enabling them to prey on relatively large prey – envenomated insect prey have been observed to be paralyzed in seconds and dead in minutes (von Reumont et al. 2014a). Furthermore, some pseudoscorpions engage in cooperative hunting of (relatively) large prey such as beetles and millipedes, for which the use of venom by multiple individuals may be necessary (von Reumont et al. 2014a).

Insects

Venom is found in several groups of insect, whether functioning primarily in predation or defense: true bugs, beetles, hymenopterans, lepidopterans, fleas, and flies. In fleas and (blood-feeding) flies, such as mosquitoes, the venom contains toxins that interfere with blood clotting and keep a steady supply of blood flowing, as well as reducing inflammatory and other such responses in the host (Andersen et al. 2007; Ribeiro et al. 2004). Such effects are typical of other venoms that act to increase ease of feeding in parasitic animals, which has led to substantial convergence in blood-feeding species (Andersen 2010).

Predatory (and defensive) venoms in insects are exceptionally diverse, but Edstrom (1992) provides good discussion of some of these. Venom use in predation (in addition to the blood-feeding flies and fleas) occurs in true bugs, beetles, and hymenopterans (Fig. 2). In hymenopterans, parasitoid wasps were highlighted earlier ("Predation" subheading in the "Venom Functions" section), but many nonparasitic wasps and ants use venom to subdue prey. For instance, most ant families use venom (a few have evolutionarily lost their venom), and predation is thought to be the main driver of its evolution (Aili et al. 2014). The venom of wasps and ants quickly immobilizes and/or kills their prey with paralyzing neurotoxins, and food is normally carried back to the nest (in both solitary and social species) to be consumed – although some solitary ants will feed where the prey is found.

Bugs represent an interesting example of venomous insects because they are characterized by piercing and sucking mouthparts (Fig. 2). This has the consequence that the carnivorous (or blood-feeding) species are prime candidates for the use of venom as their mouthparts are well structured as a venom injection apparatus. Furthermore, the necessity to feed on liquid food requires that bugs are able to liquidate their insect (or in the case of some belostomatid bugs, vertebrate) prey before consumption. Therefore, many bug venoms contain paralytic or otherwise immobilizing toxins alongside some digestive venom components (Sahayaraj and Muthukumar 2011; Zibaee et al. 2012).

Although beetles are exceptionally diverse, contain a wide range of chemical defenses, and are known to have some venomous representatives, venom appears to be either relatively rare or understudied in this group. Nevertheless, groups such as predaceous diving beetles (Dysticidae) use potent paralytic venoms to quickly incapacitate prey in their aquatic environment (Formanowicz 1982), which can include large items such as fish and amphibians. Venomous beetles also contain a

diversity of venom systems, which often includes jaws but also far more unusual strategies. For instance, in the cerambycid long-horned beetle *Onychocerus albitarsis*, the antennae have been modified into a sharp and flexible venom injection system that is used primarily to subdue prey (Berkov et al. 2008).

The use of insect venom in defense is frequent, though only lepidopterans and social hymenopterans are likely to have had their venom evolution primarily shaped by this function. In social wasps, bees, and ants, the venom often contains numerous components which cause pain and discomfort to predators, and in some species (e.g., bullet ants and fire ants), the pain generated can be excruciatingly intense (Aili et al. 2014; Edstrom 1992). Furthermore, because defensive venom evolution often occurs in social hymenopterans, multiple stings are frequent and act to increase the magnitude of the symptoms suffered by the predator.

Although many adult Lepidoptera (butterflies and moths) contain toxic defenses, venom appears to be restricted to the caterpillar stages. Furthermore, in this group, venom is entirely a defensive strategy as most species are herbivorous and venom is not known from carnivorous species (see Pierce 1995 for a review of carnivorous caterpillars). The venom system of caterpillars typically involves extremely fragile hairs which break off easily upon contact, and venom is transferred from associated glands or specialized secretory cells (Carrijo-Carvalho and Chudzinski-Tavassi 2007; Edstrom 1992). The venom of caterpillars is relatively poorly known but causes immediate pain and intense irritation, and some species are capable of interfering with blood systems and causing fatal envenomations in animals as large as humans (Carrijo-Carvalho and Chudzinski-Tavassi 2007).

Echinoderms

The venom of echinoderms (starfish and sea urchins) has evolved purely for defense – sea urchins are herbivorous grazers, and starfish feed on sessile or slow-moving organisms, and so there is little or no additional benefit to be gained from predatory venoms. Although other starfish may be venomous, they are an understudied group and only the crown-of-thorns starfish (*Acanthaster planci*) is known to use venom from dorsal spines in defense. Envenomation by this species causes a broad range of symptoms ranging from intense pain, irritation, and vomiting through to more systemic effects of hemolysis and reduction of central nervous system activity (Lee et al. 2013). In sea urchins, many species have a venom apparatus contained within specialized structures known as pedicellaria, which are distributed over the dorsal surface (Edstrom 1992). These are small structures which have three "claws," each one containing a venom gland, that are responsible for transferring venom to predators. The venom appears to act mainly as a neurotoxin, but its function against natural predators is poorly known. The best-known species, *Toxopneustes pileolus*, can cause respiratory difficulties in humans (Edstrom 1992). Furthermore, many sea urchins possess long spines which are often easily broken in the skin of predators, though only a few of these species seem to have venom glands attached to the spines – the others relying on irritation directly from the wounds (O'Neal et al. 1964).

Fish

Venom systems are highly diverse in fishes and have evolved multiple times (Smith and Wheeler 2006; Fig. 1). Representatives of venomous fish are known from both cartilaginous (in both sharks and rays) and bony fish but across the entire group function purely for defense. A defensive role is particularly important in species which are relatively slow moving or spend a great deal of time remaining still, and venomous fish often exhibit such behaviors (Edstrom 1992). The venom system itself typically consists of spines, though the precise structure varies greatly – compare, for instance, the flat and serrated spine of stingrays to the needlelike spines of stonefish. The venom of fishes remains poorly known for most species and varies greatly between species (Smith and Wheeler 2006) but often contains potent neurotoxins which induce intense pain and respiratory paralysis, as is common for defensive venoms.

Amphibians

Although many amphibians are poisonous or possess other forms of chemical defense, only two genera of salamanders and two species of frog can be considered to be venomous: the salamanders *Pleurodeles* and *Echinotriton*, and the frog species *Corythomantis greeningi* and *Aparasphenodon brunoi*. In the salamanders, the ribs are sharp-tipped and can be protruded through the skin, piercing poison glands and coating the ribs in toxins before puncturing the skin of a predator (Heiss et al. 2010). The mechanism of action remains unknown, but injection of these toxins is lethal to many potential predators including mammals and other amphibians (Heiss et al. 2010). Recently, Jared et al. (2015) described the use of venom in the two frog species. In both species, bony spines on the head are used to pierce the skin of predators and inject a potent venom into a predator. The toxins present causes intense pain, edema, and visual difficulties. Jared et al. (2015) also suggest that other frogs may possess venoms using similar spiny delivery systems, but definitive evidence is currently lacking.

Squamate Reptiles

Reptile venoms have received more study than any other venomous animal and consequently have generated several book-length treatments (e.g., Chippaux 2006; Fry 2015; Mackessy 2009), largely focused on molecular toxinology and clinical implications. Until recently, venom was considered to have evolved multiple times in snakes and once in *Heloderma* lizards, but recent work suggests that it has evolved once, early in the group containing snakes, *Heloderma*, and *Varanus* monitor lizards (Fry et al. 2006) which has subsequently been called "Toxicofera" (Fig. 2). However, it should be noted that some authors have challenged this idea and contend that the traditional view of multiple origins of venom in reptiles is correct (see Mulley et al.'s

► Chap. 4, "A Critique of the Toxicoferan Hypothesis" in this volume for further discussion). Assuming a single origin, several lineages have subsequently lost venom due to alternative adaptations that lessen the benefit of venom (Fry et al. 2013). Because almost all squamate (snake and lizard) venoms are primarily used for predation, the factors involved in the loss of venom tend to be related to alternative means to catch and subdue prey, such as constriction, or alternative diets, such as eggs or leaves, that do not require subjugation. Because of this tight link of venom to predation, diet is often the major factor influencing variation in squamate venoms (e.g., Daltry et al. 1996). Nevertheless, some groups have also had their venom evolution driven substantially by defensive function, such as spitting cobras (Young et al. 2004) and likely *Heloderma* (Beck 2005).

Archosaurs

The archosaurs (including crocodilians, dinosaurs, and birds) were traditionally considered a completely non-venomous group of animals. However, recently Gong et al. (2010) proposed that the dromaeosaurid (theropod) dinosaur *Sinornithosaurus* (and perhaps also other "raptors") was probably venomous based on the characteristics of its skull and teeth and used their venom primarily for predation. Although absolutely conclusive evidence for venom is difficult or impossible to retrieve from the fossil record, Gong et al. (2010) make a convincing argument that it was likely possessed in this and perhaps other dinosaurs in the "raptor" lineage (Dromaeosauridae).

Interestingly, this dinosaur was closely related to the early ancestors of birds, and yet despite their diversity, no birds are known to be venomous. Given the frequency of venom in other similarly diverse groups, that birds possess a number of hard parts (such as beaks and talons) that could facilitate venom delivery, and that some birds have used other toxic defenses such as poison, it is perhaps surprising that none have been found. There are many potential explanations for this, although it is difficult to discount chance as the reason. It may be that the extra weight of venom apparatus would be disfavored in flying animals or that the additional energetic expenditure of producing venom is too costly in addition to that needed for flight and high metabolism. Alternatively, it could be that most birds feed on small prey and have flight as an effective defense, therefore removing selection for venom for these two functions (although an advantage in intraspecific competition could still be beneficial for many species).

Mammals

Venom has evolved at least four times in mammals and probably more due to multiple origins within these four groups: platypuses, vampire bats, slow lorises, and insectivores. There is also some fossil evidence that some extinct mammals were also venomous (Fox and Scott 2005). Therefore, venom is rare but taxonomically dispersed in mammals. Vampire bats share similar venom characteristics with other

blood-feeding animals, with toxins acting to maintain blood flow (via anticoagulant effects and vasodilation) and avoid disturbing (in this case waking) the host by reducing pain and inflammation (Ligabue-Braun et al. 2012; Low et al. 2013).

There are two mammals which use venom for intraspecific competition in addition to defense: platypuses (Whittington et al. 2009) and slow lorises (Nekaris et al. 2013). Although sharing function, these two venoms seem to act in different ways. Slow loris venom causes pain, inflammation, and dramatic tissue destruction in conspecifics (Nekaris et al. 2013), which may give a longer-lasting advantage to the inflicting male or may simply be a corollary of the suspected multifunctionality of slow loris venom. The venom system is highly unusual in that the venom is formed by mixing two nontoxic fluids, brachial gland secretion from near the elbow and saliva, which combine to form a toxic venom which is injected by biting (Nekaris et al. 2013). In platypuses, the venom is less destructive but still inflicts a great deal of pain and inflammation (Whittington et al. 2009). The venom is delivered via spurs on the hind legs, though there are sex and seasonal differences in the venom system. Female platypuses lose the spurs early in life and generally have a degenerate venom system; males keep the spurs but the venom gland only becomes highly active during the breeding season (Ligabue-Braun et al. 2012). These changes strongly suggest that intraspecific competition is the main driver of venom evolution in platypuses, even if it is also occasionally used in defense.

Several groups of insectivores have either been demonstrated or suspected of using venom to assist predation. At least three shrews (*Blarina* and *Neomys*) are known to be venomous, as are solenodons, all of which have venom glands in the lower jaws and transfer venom to prey via a bite, and there is some evidence that other shrews are similar (Ligabue-Braun et al. 2012). Moles are also strongly suspected of being venomous as they have similar glands in the lower jaw and are known to store paralyzed worms in burrows (Ligabue-Braun et al. 2012). It seems to be generally the case for insectivores that the venom does not usually kill prey but rather immobilizes it in a live but paralyzed state, in which it is stored for later consumption.

Evolutionary Drivers of Variation in Venoms Between and Within Species

Venoms are highly variable both between species and within a single species. The evolutionary causes of this variation will vary depending on the primary function(s) of the venom, and the extent of the variation will partly depend on any constraints acting on the system. Although this section will focus on the generation and maintenance of variation, it is worth mentioning the influence of convergent evolution in constraining diversity of toxins. Convergence is a common theme in venom evolution (e.g., Casewell et al. 2013; Fry 2015) and can be seen at two levels.

The first is at the level of individual toxins, wherein the same protein structures are repeatedly altered to function as toxins across the animal kingdom (Fry et al. 2009a, c). This is likely a consequence of a combination of similar basic protein structures being available as body proteins to many different animals, therefore the

raw materials are similar before toxin evolution, and that toxin evolution by small alterations of particular physiologically active molecules is likely to be easier to achieve as the molecule is already adapted to interact with physiological systems.

The second level that convergence can be seen is in whole venoms, by which the author means that venom in some form has evolved repeatedly a large number of times across the animal kingdom (Fig. 1). This level could be extended to the consideration that venom functions have also evolved convergently throughout the animal kingdom, especially predatory and defensive venoms (but use in intraspecific competition has evolved at least in monotremes and primates).

It should also be noted that venom is an energetically expensive product (McCue 2006) as so selection would be expected to act to optimize the cost-benefit ratio of the functions for which it is used. Therefore, venom evolution has followed a complex path of diversification and convergence which has shaped the observed variation in animals.

Interspecific Variation

The chemical composition of venom often varies remarkably between even closely related species. For predatory venoms, different species may have different diets, and this may drive divergence in the venom of each as it increases the efficacy of the venom toward that species' particular prey (Barlow et al. 2009). It is highly likely that such dietary shifts are the main selection pressure driving variation in predatory venoms as there are numerous observations of venom systems degenerating (presumably to save energy) when diet changes make venom unnecessary (e.g., Fry et al. 2013).

For defensive venoms, there is little clear evidence that different predator communities drive differences in venom. However, this would be difficult to obtain for multiple reasons. Firstly, it is often unknown what the actual predator community is in a given area for a given venomous prey species. Secondly, predators are likely to be attacking multiple prey species, and perhaps multiple venomous prey species, and so attributing changes to a particular predator is difficult. Thirdly, venoms (including defensive venoms) are often effective against a wide range of predators so even if the predator community were fully replaced, the same venom may still be effective. In other chemical defense systems, it seems that the defense is readily gained but difficult to lose which suggests that there are strong and general individual benefits (Arbuckle and Speed 2015). Furthermore, natural enemies such as predators are expected to impose strong selection on defenses, and therefore it is likely that predators do drive variation in defensive venom, but it may be at a broader scale that is typically examined.

Competitive and reproductive venoms are so poorly understood that the drivers of their variation between (or within) species are unknown. For venoms with a reproductive function, we may expect that sexual conflict is strong as males chemically manipulate females and females may be selected to resist this. Consequently, we might expect that variation in venoms between closely related species using venom in reproductive interactions is much higher than otherwise expected, but this remains to be investigated.

Finally, environmental drivers of interspecific variation in venoms are understudied (excepting prey choice and availability). However, we might expect, for example, that predatory venoms may be more potent in species that hunt in environments where prey may escape out of reach unless venom takes effect especially quickly, such as in slow-moving aquatic predators or those hunting in dense habitats or where prey can escape to burrows.

Intraspecific Variation

The same drivers of interspecific variation may also drive intraspecific differences between populations, but there are other considerations that are specific to the latter. However, many of these are not evolutionary in origin. For instance, variation in venom can be a consequence of amount of energy available to an individual for toxin manufacture, or time since last envenomation as venom supplies need to be replenished (and different toxins may regenerate at different rates).

Other causes of intraspecific variation may be a consequence of evolution. For instance, sex differences in venom may reflect sex differences in diet or predation risk (e.g., in *Bothrops jararaca*; Furtado et al. 2006), in which niche partitioning between sexes leads to feeding on different prey types and consequent shifts in venom, increasing variation within the species. Similarly, age-related variation may be a consequence of diet or predation differences coupled with a smaller venom yield in smaller individuals (e.g., in *Crotalus* spp.; Furtado et al. 2003; Mackessy 1988). This situation allows young, and therefore small, individuals to possess a relatively more effective (e.g., higher toxicity) venom that could offset the low venom yield available to secure prey or repel predators.

Finally, in predatory venoms, prey populations or communities may change over time. This could conceivably generate selection on venomous predators to have a quick evolutionary response in their venoms, leading to increased mutation rates in venom genes compared to other genes (either in coding or regulatory regions controlling expression of different components). This situation would lead to high variation in venoms within a species, and within populations, despite the selection acting on evolvability rather than favoring the increased variation per se. Nevertheless, evidence for this scenario is currently lacking and remains a mere possibility, though if true it could provide an additional explanation for many toxins being part of multigene families.

Antagonistic Coevolutionary Interactions Are the Common Thread in Venom Evolution

All venoms have evolved in the context of antagonistic coevolution. Despite the massive diversity of venoms, venom delivery systems, venom functions, and venomous animals, this is one key point which is applicable throughout. The idea of the "arms race" is well known in natural enemy interactions such as predator-prey and

host-parasite systems (e.g., Endler 1991) but is less well appreciated as a core concept in venom evolution throughout the tree of life. The antagonists may be traditional natural enemies (e.g., predator-prey, host-parasite) as in the case of predatory and defensive venoms or less traditional (in this context) such as conspecifics of the same or opposite sex as in the case of competitive or reproductive venom functions. Nevertheless, because they all represent parties with opposing interests (one side wants to envenomate, the other wants to avoid envenomation), there are implications for considerations of the evolution of venomous animals: it never takes place in isolation.

In essence, any evolutionary change in venom – whether gain, loss, or alteration – will impose selection pressure on another organism to limit or remove the benefits conferred to the venomous animal. This will both lead to diversification of the venom as the arms race forces both parties to continually adapt but also constrain the advantages that can be gained. A common example of coevolution in the context of venomous animals is venom resistance. Predators that eat venomous prey have often evolved resistance to the prey's venom to enable consumption (Drabeck et al. 2015). Similarly, prey that are eaten by venomous predators often have a high level of resistance to the predator's venom (Heatwole and Poran 1995; Heatwole and Powell 1998). In some cases, prey species are more resistant to the venom of their predators than to other venomous predators that do not eat that species – a result that would be unintuitive without an appreciation of coevolution. This concept is taken further by nudibranch mollusks which feed on venomous cnidarians but are able to not only avoid envenomation but extract the entire venom system from the cnidarian and transport it to the nudibranch's surface for its own defense (Greenwood 2009).

Ecological and Evolutionary Consequences of Venom (and Other Chemical Defenses)

Natural enemy interactions are expected to lead to phenotypic divergence and evolutionary diversification in organisms, as a consequence of the coevolutionary arms races that they fuel (Ehrlich and Raven 1964; Vamosi 2005). In essence, the expectation is that effective antipredator defenses should lead to a greater freedom of movement (without having to be as cautious about potential predators) and hence occupy a broader niche space. The broader niche space may allow more opportunities for diversification, and the arms race itself is predicted to generate evolutionary diversification in such scenarios (Ehrlich and Raven 1964) – a phenomenon known as "escape-and-radiate" theory.

Note that these predictions stem from theories of defense but should apply well to venomous animals since most use venom either primarily or secondarily in defense. Unfortunately, little research effort has been focused on the evolutionary and ecological consequences of possessing venom in animals, and so this discussion will borrow from the literature on other chemical defenses. However, because the predictions are based on the interplay between the predators and the repellent nature of the defense, the response should be similar in many cases across specific forms of defense.

Higher predation risk (and correlates of higher predation risk) tends to favor the evolution of effective defenses, but the consequences to ecology and life history of the animals after the evolution of venom are less well known. Two general points are of particular interest though. The first is that chemical defense does seem to be associated with a broader niche space, as predicted above (Arbuckle et al. 2013). Specifically, in musteloid mammals (a group including skunks, badgers, and otters), those that use repugnant anal gland secretions in defense had a less constrained activity period and a broader diet. The second point is that chemical defense is associated with slower life histories including such traits as longer life span (Hossie et al. 2013) – although venoms used primarily for predation did not show the same pattern of longevity, suggesting specificity to defensive venoms. This is expected based on evolutionary theories of the life history of aging that predict slower senescence and generally slower life histories in species with lower extrinsic mortality, such as from predation (Blanco and Sherman 2005).

Few empirical tests of the prediction from "escape-and-radiate" theory, that chemically defended animals should have higher diversification rates, have been conducted. Recently, Arbuckle and Speed (2015) investigated this idea using amphibians and found that chemically defended lineages actually had lower diversification rates, due to an increased extinction rate. The raised extinction rate was also observed in present-day amphibians Arbuckle (2015), wherein chemically defended species are more likely to be threatened (based on IUCN Red List conservation status) than non-defended species. The most plausible mechanism to explain this is that because chemically defended species should have slower life histories, they should be less resilient to population declines due to slower rates of subsequent population increase.

Conclusion and Future Directions

Venom has evolved frequently across the tree of life and is consequently found in many disparate groups of animals. The benefits obtained by venomous animals are most often related to enhancing prey capture or avoiding attack by predators but can include other aspects of biology such as competition and reproduction. These functions are not mutually exclusive, but all take place in the context of antagonistic coevolutionary interactions – perhaps the one comprehensive rule of venom evolution. Venom displays extensive variation both within and between species, which can be driven by various processes relating to the functions of the particular venom. Finally, the evolution of venom, especially as a defensive trait, can have important long-term consequences for the ecology, evolution, and conservation of venomous animals.

Throughout this chapter, many gaps in our knowledge have been highlighted. However, perhaps the most promising for future work falls into the following two areas. Firstly, there are many venomous animal groups which have been given very little attention, particularly among the invertebrates, and directed research into those groups would provide insights into the evolution and diversity of venom, as well as

uncover novel toxins which could potentially yield a multitude of new pharmaceutical products. Secondly, the macroevolutionary consequences of venoms have been almost ignored until very recently, yet provide an opportunity to understand how venomous animals originated and how their future is likely to play out. These areas are likely to be extremely fruitful for further investigation.

Cross-References

- ▶ A Critique of the Toxicoferan Hypothesis
- ▶ Evolution of Resistance to Toxins in Prey
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

2

Anita Malhotra

Abstract

Toxins represent one of the fastest evolving types of protein to be found in animal systems, sharing many of their features with other protein families that respond to extrinsic factors, such as those involved in immunity, and detecting and responding to the environment in which they live. However, studies on toxin genes have been lagging behind those on other gene families as until very recently, no fully sequenced genomes from venomous animals have been available. In this chapter, the molecular forces acting on toxin gene sequences are compared to those acting on other non-toxin genes, addressing in particular several features that have been stressed in the toxinological literature, i.e., their hypervariability, accelerated evolution, and apparent focal mutagenesis centering on the active site of the toxins. The accepted paradigm that the birth-and-death model underlies toxin multigene family evolution is challenged by studies that show both concerted evolution and birth-and-death can give rise to similar patterns following gene duplication and that both models may operate simultaneously. Much of the dynamics of gene duplication and the fate of duplicated genes seem to depend on the genomic and biological context in which they occur. Therefore, there is no reason to expect toxin-encoding genes from diverse animal groups to show common mechanisms of evolution.

Keywords

Venomous animals • Toxin evolution • Toxin gene • Gene duplication • Gene Conversion

Contents

A. Malhotra (✉)
School of Biological Sciences, Bangor University, Bangor, Gwynedd, UK
e-mail: a.malhotra@bangor.ac.uk

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_5

Introduction

A major endeavor of evolutionary biologists is to understand variability in rates of evolution of different classes of genes (Hirsh and Fraser 2001). Toxin evolution represents a unique example of protein evolution with applicability to an increasingly broad range of venomous taxa (Casewell et al. 2013; von Reumont et al. 2014) but also to other classes of rapidly evolving genes in most organisms, such as those involved in the response to external stimuli (Bowmaker and Hunt 2006; Niimura 2012). Although Brookfield (2000) cautions that fast-evolving is not necessarily equivalent to evolving under positive selection, Endo et al. (1996) identified neurotoxins from snakes among only 17 out of almost 4000 gene groups surveyed, which showed evidence of being under positive selection. To what extent are the mechanisms operating on proteins expressed in venom glands similar to those acting on proteins expressed in other body tissues? The purpose of this chapter is to review the features of toxin evolution in the context of recent findings about protein evolution in general.

Dating from the 1970s (Hirsh and Fraser 2001), the predominant view of protein evolution has been that the conservation of protein sequence is largely determined by the dispensability of the protein in biochemical networks. However, this largely applies to single copy genes, which are more easily studied, and gene duplication can act as a trigger for innovation in protein function. For example, Hahn et al. (2007) provided evidence for excess positive selection on coding sequence gene families that had experienced rapid expansion in primates. Other factors affecting the tendency to evolve fast may include differences in expression level (Reyes-Velasco et al. 2015), number of duplicate copies, and rates of gene turnover within duplicated gene families (Katju and Bergthorsson 2013). In addition, particularly pertinent to tissue-specific expression as seen in toxins that are expressed only in the venom gland, a major cause of evolutionary innovation is expression shifts caused by mutations in regulatory noncoding regions (Margres et al. 2015).

The main features of toxin families highlighted in the extensive literature on this topic are their hypervariability (Conticello et al. 2001; Zhang et al. 2014), signature of strong positive selection (often referred to as accelerated or Darwinian evolution), an excess of non-synonymous substitutions compared to synonymous substitutions, and the nonuniform distribution of mutations over the length of the protein sequence. These are the features on which the following chapter will focus, followed by a discussion of evolutionary phenomena seen in other similar gene families and the extent to which they might apply to toxin gene families. The recent inclusion of venomous animals into the select group of organisms that have had their genomes sequenced may soon allow some outstanding questions about toxin evolution to be answered.

Positive Selection/Accelerated Evolution

As toxinologists and molecular evolutionists have described for over two decades, a common feature of toxin genes is that the exons are considerably more variable than the introns (Ogawa et al. 1996), in direct contrast to the usual observations for proteins. Moreover, the exons frequently show a signal of positive selection in that non-synonymous substitutions (K_A, sometimes written as dN) occur more often than expected under the null hypothesis of neutral evolution (where the number of non-synonymous and synonymous substitutions, K_S or dS, would be expected to be equal).

However, excessive reliance on between-species K_A/K_S tests for detecting selection has been highlighted by Brookfield (2000). A high K_A/K_S ratio, indicating positive selection, might be found where substitution is actually neutral (McAllister and McVean 2000), and a ratio less than one, which might be taken to indicate purifying selection, may instead be the result of adaptive substitution in some regions of the protein being masked by purifying selection in others. Indeed, Bazykin and Kondrashov (2012) have shown that, in the *Drosophila* genome, the strongest positive selection observed is that which drives allele replacements at conservative sites, accelerating evolution by a factor of approximately 40, as opposed to a factor of approximately 5 at rapidly evolving sites.

A less frequently used alternative to the K_A/K_S tests for detecting selection are those that rely on examination of within-species polymorphism in comparison to between-species fixed differences, such as the McDonald-Kreitman test (McDonald and Kreitman 1991). This test has been applied to a wide range of multigene families in plants and animals and had been extended to account for background selection and selective sweeps that may affect genome-wide patterns of polymorphism (Messer and Petrov 2013).

Another complicating factor is that selection is likely to be episodic and may even change direction over time. It has to be borne in mind that the result of positive directional selection is to drive a rare allele toward increasing in frequency at the expense of the existing most frequent allele, while purifying selection will favor the most frequent existing allele against newly introduced rare alleles. Thus, directional selection may only act when changing conditions favor switching or tweaking of toxin targets. The effectiveness of the process is linked to the effective population size, as in small populations the chances of fixation or elimination by random fluctuation are higher than in large ones. Luckily, more biologically realistic methods that are capable of detecting such episodes of selection at individual sites and/or lineages have been developed and are being constantly refined (e.g., the plethora of methods available in the HyPhy package (Kosakovsky Pond et al. 2005; Murrell et al. 2015)) and are now being applied to toxin evolution (Sunagar et al. 2015).

Evolution of Hypervariability

Ever more sensitive methods of studying the small amounts of venom produced by individual specimens of some species rather than traditional pooled venom approaches, for example, have revealed that each individual *Conus* may be

synthesizing up to 1000 bioactive peptides, and there may be virtually no overlap between species and even rather little overlap between individuals of the same species (Dutertre et al. 2010). When the diversity of venomous animals is taken into account (over 700 species of *Conus* alone), the true number of toxins generated by animals is staggering. This hyperdiversity is not restricted to toxin genes but applies to any genes whose products interact with other species, whether as predators, prey, or pathogens. This panel of interacting organisms is likely to be different for each species or even populations within species, and thus genes of this type are likely to be exposed to different selection pressures on a regular basis. As a result, they are often hyperdiverse, encoding hundreds if not thousands of different variants within the same species or even individual. Classic examples are the major histocompatibility complex (MHC) (Bernatchez and Landry 2003) and defensin genes (Das et al. 2010) in vertebrates and surface antigens of microbial pathogens (Zilversmit et al. 2013), which are involved in evading the innate immune response of their hosts. Other examples include plant genes involved in pathogen resistance (Bergelson et al. 2001) and proteins involved in sensing of the environment, such as visual (Bowmaker and Hunt 2006) and olfactory genes (Niimura 2012), and in communication (Wilburn et al. 2012). As well as its practical relevance, this hyperdiversity raises some very interesting theoretical questions. How is it generated and maintained? How is the expression of particular toxin variants controlled?

Many toxins belong to multigene families (MGF), in which genes (often coding for compounds performing a basic physiological function in the organism originally) are duplicated and inserted into the genome (Wong and Belov 2012). Gene duplication has been recognized as an important source of evolutionary innovation in eukaryotes for decades, and recent work suggests that it may have been fundamental to the successful radiation of early eukaryotes (Zhou et al. 2010). The original model proposed to explain the contribution of gene duplication to evolution was that the presence of a duplicate copy of a gene allowed the development of new functions as it was free from functional constraint (neofunctionalization model). However, there are a number of problems with this model, outlined by Bergthorsson et al. (2007), largely relating to the fate of the nonfunctional copy while free from selection. While purifying selection may act on a duplicated gene to maintain it in the population, its ability to acquire new functions would then be limited. However, the acquisition of new functions through random mutation while remaining free from more common deleterious mutations and avoiding elimination from the population through drift would seem to require unrealistically large populations. This has been confirmed by simulation studies using point mutation as the predominant process involved in change and assuming that the new function requires more than one mutation. However, other, more complex, processes by which a larger number of changes may occur in a single step are also known to occur, including recombination and insertion-deletion events (see section "Concerted Evolution by Gene Conversion" below).

A number of variants of the basic model have been proposed (reviewed by Innan and Kondrashov 2010) which differ, sometimes rather subtly, in aspects such as the type and timing of selection acting on one or both of the duplicated genes, during the

process of spread and fixation in the population. Despite this, Innan and Kondrashov (2010) pointed out that most of the critical information that would allow one model to be favored over others come from the early stages of the process when distinguishing different types of relationship between duplicated copies, which are essential for proper understanding of the evolutionary dynamics, can be difficult (Mendivil Ramos and Ferrier 2012). While phylogenetic methods (e.g., Han et al. 2009) may provide greater power to distinguish recent paralogs, lineage-specific duplications may still represent relatively old events if the lineages diverged a long time ago and duplication rates are high. In addition, phylogenetic methods depend quite heavily on the accuracy of the phylogenetic hypothesis being used. Moreover, such studies often rely on analyzing protein or complementary deoxyribonucleic acid (cDNA) sequence of toxins, rather than gene sequences, as this is still more readily available than genomic data. However, if protein products are exposed to strong positive selection, phylogenetic analysis on coding regions may well give misleading results about the relationships of the genes themselves (Malhotra et al., 2015). The large size of such datasets also frequently leads to simplification of phylogeny reconstruction methods. As a consequence, complex evolutionary phenomena such as recombination and rate variation (both among-site and among-lineage) may not be adequately controlled (Arenas 2015). The incorporation of biologically reasonable variation in processes among sites may often account for the apparent derived trends predicted by simpler methods (Goldstein and Pollock 2006). Further, rate calculations depend on the availability of known divergence times among taxa. As a result, few studies of toxin families have attempted to quantify duplication rates and those that have usually employ multiple assumptions to produce a range of rates (Binford et al. 2009). However, it seems highly probable that toxin genes are evolving at some of the fastest rates yet recorded (Chang and Duda 2012).

The "birth-and-death" model of gene evolution (Eirín-López et al. 2012) was first proposed in relation to MHC genes, and this model has been applied frequently to the evolution of specific gene families thereafter. Birth of new genes is relatively easy to observe, but death (gene loss through deletion or pseudogenization) is far less so. Gene loss is often ignored as it is assumed that most duplications will in fact be deleted as they are more likely to be slightly deleterious than beneficial. However, gene loss may play an important role in reshaping the venom arsenal of similar organisms (Rachamim et al. 2015). Additionally, when it results from pseudogenization rather than deletion from the genome, it may in fact still provide fodder for further evolutionary change, as several studies have now indicated that pseudogenes retain the potential to become new genes (Balakirev and Ayala 2003; Duda and Remigio 2008).

Although the sequencing of more and more genomes will make the job of studying gene gain and loss much more robust, presently, gene loss can only be predicted by analytical methods such as gene-species tree reconciliation (Szöllősi et al. 2015), which may suffer from errors and, in large gene families, cannot distinguish between unsampled genes and genes which have really been lost from the species. There may also be hidden genes, those that are still functional but are not

normally transcribed. Casewell et al. (2014) suggested that between 44% and 70% of toxin genes may not be transcribed, although this is complicated by possible translational controls on functional genes as well.

Nevertheless, some carefully conducted studies (e.g., Chang and Duda 2012) have supported the hypothesis of relatively constant and high rates of gene turnover in toxin gene families. This process would provide a constant supply of new genes, ready to undergo selection for new functions, which is likely to far outstrip the rate of novel mutation (Katju and Bergthorsson 2013). In fact, the number of gene copies circulating in a population at any one time is likely to be much higher than estimated, since phylogenetic analyses cannot separate the rate of duplication of genes and the rate of their subsequent fixation in the population, and instead represents a combination of both these processes (Innan and Kondrashov 2010). It is also likely that a greater number of duplicate copies coding for a particularly important toxin in the venom might in itself be beneficial, since snakes need to rapidly replenish the venom contained in the lumen of the venom gland once it has been expended, and the rate of transcription will be limited by the number of genes encoding the toxin. In other words, gene dosage effects might be beneficial rather than detrimental in this case (Kondrashov 2012). However, it is not yet very clear whether this would provide a temporary advantage only, with the duplicates being lost once the advantage of that particular isoform is lost (e.g., by a change in diet), or whether these duplicates then provide fodder for further functional divergence (Katju and Bergthorsson 2013). More recent studies have provided evidence for the latter in genes that have environmental response functions (Chain et al. 2014).

Some evidence to support the importance of gene dosage in the preservation of duplicated toxin genes has been found in venomous snakes. Malhotra et al. (2013) found a number of distinct gene copies encoding the same protein, which in all cases corresponded to the most abundant isoform detected in the venom by mass spectrometry, in a study of phospholipase A2 (PLA2) evolution in pit vipers. Oguiura et al. (2009) found up to 32 copies of genes encoding crotamine in *Crotalus durissus* per haploid genome and detected a positive correlation between the number of copies of the *crotamine* gene and the concentration of crotamine in the venom. Even a slight advantage provided by overexpression of particular toxin variants might be enough for selection to be the primary force acting to fix a new duplicate in the population, rather than neutral drift. Bergthorsson et al. (2007) also pointed out that genes often possess auxiliary functions when present in high copy number that are not seen when present in low copy number. Although evidence for this is mostly from prokaryotes, it has been shown that a given snake venom protease may behave both as an anticoagulant and coagulant, depending on its concentration in the venom (Matsui et al. 2000).

Snake venom PLA2 evolution was one of the case studies cited in defense of the innovation-amplification-divergence (IAD) model (Fig. 1) developed by Bergthorsson et al. (2007). The IAD model additionally predicts that the emergence of a highly functional allele with a novel function would lead to removal of selection

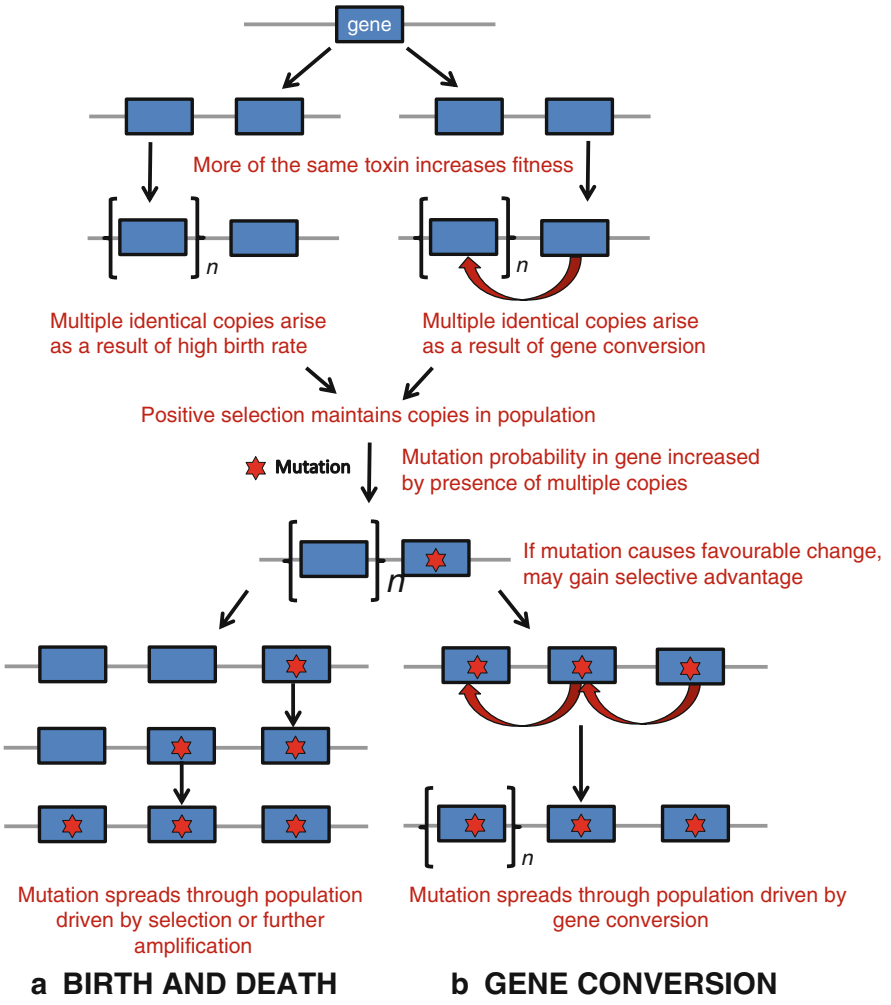

Fig. 1 The fate of duplicated genes depends on a complex interplay of processes that tends to fix them in, or eliminate them from, the population (including drift, selection, pseudogenization, and gene conversion). One reason that duplicates might be maintained in the population by selection is through the dosage effect, if an increased amount of gene product (e.g., a toxin that is particularly effective at subduing a commonly available prey type) provides a fitness benefit to the organism. The larger number of copies then provides an increased target for point mutation, which occurs much more slowly. However, a similar pattern may be produced by both a birth-and-death model with a rapid rate of gene duplication (**a**) and gene conversion (**b**) when the presence of additional gene copies similar to the advantageous one is favored by selection, although the probability and speed of fixation of a favorable mutation in the population will be faster in the case of concerted evolution when direct selection on gene duplicates is weak. Gene conversion is also known to maintain multiple gene copies that are already fixed in the population, with or without selection favoring these duplicates.

on paralogous copies with increased loss through pseudogenization or deletion. This model may help to explain the "streamlining" of venom that has recently been reported from sea snakes (Pahari et al. 2007) and cone snails (Duda and Remigio 2008), where a relationship between specialization of diet and the number of toxin isoforms expressed in individual venoms was observed. In addition, it may also be applied to the observed restriction of PLA2 isoform expression in populations of Taiwanese *Viridovipera stejnegeri* that are known to feed on more challenging prey than frogs (Creer et al. 2003). These patterns could result from increased levels of purifying selection on mutations which take the "adaptive" isoform away from its optimal form, or, conversely, relaxation of positive selection in the case of moving to a less challenging prey item.

Many studies of toxin families in a diverse range of organisms have also provided evidence that adaptive molecular evolution is directed toward the active site of toxins, regions that are implicated in protein-protein interactions, which are usually located on the surface of the protein (Casewell et al. 2013). Conversely, purifying selection is usually detected in residues important for maintaining the structural stability of the protein. In large proteins, such as snake venom PLA2s (Kini and Chan 1999), these may form a conserved core, while in smaller toxins, they are surface-exposed residues which are clustered on the opposite side to the active surface (Kozminsky-Atias and Zilberberg 2012). It is often not stated explicitly what mechanism might bring about this pattern. Most authors implicitly refer to a mechanism whereby random mutations occurring in active sites become fixed more rapidly than random mutations occurring elsewhere due to the action of positive selection when they confer an advantage, such as the ability to subdue a novel prey type. However, directed mutagenesis, whereby the mutation rate is hypothesized to vary according to the conformational position of the residues, is also implicitly or explicitly invoked in many discussions. This is based on the apparent adaptive mutability observed in bacterial experimental systems (Bergthorsson et al. 2007), which has been a subject of intense debate and controversy for decades. It is now thought that the original "adaptive mutability" experiment can be explained by the presence of multiple copies of plasmids carrying the mutant allele (Sano et al. 2014), without the need to invoke the action of error-prone polymerases (which moreover, have never been found in eukaryotes). Thus, explanations of variation in amount of change observed at different sites must be based on varying rates of fixation in the population or species, rather than varying rates of mutation in individual genomes.

Genomic Location

One possible explanation for rapid gene turnover rates is the physical location of the genes in question (i.e., among the chromosomes). It has been observed that many hypervariable gene families in primates, which are evolving in a similar birth-and-death manner, are located in subtelomeric regions of chromosomes. In primates these regions are known to be subjected to higher rates of recombination, duplication, translocation, and other diversifying phenomena than other chromosomal regions

(Das et al. 2010; Linardopoulou et al. 2005). At present, we only have a few clues about the role that this might play in toxin evolution. Recently, Jiang et al. (2011) identified five putative tandem duplicates of three-finger toxin genes in *Bungarus multicinctus* and seven in *Naja atra*, while Ikeda et al. (2010) demonstrated the presence of five tandemly arranged PLA_2 genes (including two pseudogenes) located in a microchromosome in the Japanese habu *Protobothrops flavoviridis*. The location could be significant as reptilian microchromosomes show high rates of recombination leading to tandem duplications arising frequently (Janes et al. 2010).

Concerted Evolution by Gene Conversion

One major model of evolutionary change that has not yet been mentioned is concerted evolution. In contrast to the birth-and-death model, in this model members of a gene family tend to be more similar to each other within a species than they are between species. This is because gene conversion acts as a homogenizing factor (acting via homologous recombination-based repair of double-strand breaks transferring genetic information among paralogs or unequal crossing-over events during meiosis between tandemly arranged copies) within a population or species. Although the evidence to date from toxin gene families has favored the birth-and-death model, this might be because sampling has been biased toward interspecific rather than intraspecific comparisons and is usually incomplete to an unknown extent. Moreover, few studies have examined polymorphisms within species (Oguiura et al. 2009). More recent evidence suggests that these two models are not mutually exclusive, and mixed-effect evolution of certain large gene families, such as 5S ribosomal RNA, has been proposed (Eirín-López et al. 2012). Gene conversion has been shown to be active in large gene families and those where increased gene dosage is advantageous, meaning that quite sophisticated analyses are required to distinguish the underlying model in such cases. This suggests that future studies of toxin family evolution should not automatically assume that birth-and-death evolution is the force in action without explicitly considering concerted evolution through gene conversion (Arguello and Connallon 2011). Ignoring gene conversion when it has occurred will lead to misinterpretation of temporal patterns of selection and an overestimation of the rates of duplication.

Conclusion and Future Directions

Although the evolution of toxins is often discussed as if they were a homogenous group of biomolecules, they are of course very diverse in their structural features and genomic backgrounds. Casewell et al. (2014) highlighted that some toxin families, such as cysteine-rich secretory proteins (CRISP), and L-amino acid oxidase (LAAO) show very different features to PLA2s, snake venom metalloproteinases (SVMP), three-finger toxins (3FTx), and other toxins that are encoded by multigene families with high number of paralogs. Instead, they appear to be subject to considerable

control at transcriptional and translational stages, as well as being different in their evolutionary dynamics as revealed in the genome of the king cobra (Vonk et al. 2013). Posttranslational processes, such as proteolytic cleavage to produce multiple toxins from a single gene product, are also much more common in some gene families (such as serine proteases and SVMP) than others (such as PLA2) and are bound to considerably affect their evolutionary dynamics. Notably, Casewell et al (2014) showed that several highly transcribed genes (including SVMP and C-type lectins, which belong to generally highly expressed protein families in the venom gland of the *Echis* species under investigation) were present at very low levels in the proteome. Durban et al. (2013) also showed, in relation to ontogenetic changes in the venom of *Crotalus simus*, that a single transcriptome could give rise to distinctly different proteomes with drastically different functional capabilities and provided evidence that this was mediated by micro-ribonucleic acids (miRNA). They may also play a similar role in modulating the plastic response of adult snakes to changing environmental conditions.

Moreover, work on other, non-toxin, gene families shows that genomic architecture and signaling pathways, which may vary dramatically across the range of venomous taxa, can influence the evolutionary dynamics substantially. For example, Nozawa and Nei (2007) showed that olfactory receptor (OR) genes show very different patterns in *Drosophila* compared to mammals, which they attributed to differences in expression patterns of OR genes in the two groups, as well as more flexible signaling pathways involving ORs in mammals. Transcription factors (proteins which bind to deoxyribonucleic acid (DNA) thus affecting their expression patterns) have been shown to display lineage-specific expansion associated with adaptive niche changes in various groups including Archaea and primates (Iskow et al. 2012). The dynamic evolution of conotoxin expression patterns is also responsible, at least partly, for the differentiation of venoms of *Conus* (Duda and Remigio 2008), and Dutertre et al. (2014) recently demonstrated that the genetic complement of conopeptides can be combined very differently in different parts of the *Conus* venom gland to produce two distinct types of venom that are deployed differentially in defensive and predatory contexts. While the venom gland and the neighboring accessory gland in snakes appear to make different contributions to the venom (Vonk et al. 2013), the role of the accessory gland secretion is not yet entirely clear. It may be that in snakes, venom plays a lesser role in defense as snakes display much more flexible and varied, context-dependent, antipredator responses (Llewelyn et al. 2010). Thus, it is apparent that there may be sound biological reasons for not over-generalizing across toxin groups and venomous animals. Toxinologists will, in the future, need to examine the evolutionary history of toxins in different animal groups in much more detail, making full use of the novel analytical tools now available, to fully understand the forces which have shaped the generation of these highly sophisticated bioweapons. Luckily, rapidly advancing techniques and tools, from next-generation sequencing to large increases in computational power, that make assumption-free analysis more readily available, will assist in addressing this challenging task in the near future (Wong and Belov 2012).

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Evolution of Resistance to Toxins in Prey

3

Thomas M. McCabe and Stephen P. Mackessy

Abstract

Venoms, as simple to complex mixtures of toxic components, are well understood to be used as trophic weapons by a range of predator species. Ecological predictions obviate the response of putative prey species against predator attacks, such as the development of biochemical defenses that allow prey species to evade predation, namely, resistance. Current hypothetical predictions indicate that venom toxicity and resistance form an antagonistic dyad that may be described as a coevolutionary chemical arms race. The development of resistance in prey populations is expected to drive the evolution of novel toxicities in predator populations and vice versa, given that predator-prey pairs are stably associated through evolutionary time. The utility of a chemical arms race model to describe toxicity-resistance systems as well as known information about natural resistance mechanisms derived against venomous predators are discussed across prey species of a wide range of venomous predators. The efficacy of resistance, mechanism(s) of resistance, phylogenetic breadth of resistance, and phylogeographic distribution of resistance are provided where information is available. For many predator groups, known prey resistance is not well described, and we discuss the cause(s) of such a gap in understanding, as well as future directions for resistance research and application of known resistance information for practical and theoretical purposes.

Keywords

Predator prey interactions • Resistance • Mechanism • Evolution • Chemical arms race

T.M. McCabe (✉) • S.P. Mackessy
School of Biological Sciences, University of Northern Colorado, Greeley, CO, USA
E-Mail: thomas.mccabe@unco.edu; stephen.mackessy@unco.edu

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_6

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Introduction

Venoms are simple to complex mixtures of toxic components that are conveyed through specialized delivery systems to subdue prey (Mackessy 2002, 2010), and possibly to aid in predigestion of prey tissues (Pough and Groves 1983; Mackessy 1988). For prey species, on the defensive side of the predator/prey dyad, becoming a meal greatly decreases lifetime fitness, and predictably many forms of predator evasion have been documented. This essay discusses the nature of chemical defenses against predator venoms, often described as venom resistance, that have arisen in response to the selective pressure imposed by the chemical weapons of predators. For the purpose of this discussion, venom resistance is defined as the endogenous chemical/physiological capacity of a prey species to prevent or hinder the pathologic consequences of envenomation by a predator species. By this definition, in the absence of resistance mechanisms, venoms are pathological to prey species. This venom antagonism is in contrast to cases where a predator's venom has no bioactive effect on one or a group of potential prey species, but may be lethal to other species or groups of species (i.e., prey-specific venoms: Heyborne and Mackessy 2013; Mackessy and Saviola 2016; Pawlak et al. 2006, 2009). Venoms represent complex molecular weapons to defend against, and venom resistance is assumed to be conferred by venom-resistant molecules or mechanisms that are able to neutralize partially or fully the negative effects of a venom and its toxic constituents. Successful resistance should allow prey species to evade capture and digestion. There is evidence that in some cases, chemical neutralization of venomous components may not be sufficient to allow prey species to escape predator behaviors that enable prey capture, regardless of the effectiveness of venoms. However, behavioral responses that allow prey species to evade predators, or allow predators to successfully capture prey species, independent of the role of venom, will not be discussed.

This chapter focuses on known cases of prey resistance to predator venoms. Resistance in some groups, such as prey species of venomous snakes, is well described, but resistance in other groups, such as prey species of venomous insects,

is not well understood, and little information appears to be available even after extensive literature searches. Instances of resistance are discussed in relation to the venoms they are able to neutralize. Each section provides information regarding efficacy of resistance, mechanism(s) of resistance, phylogenetic breadth of resistance, phylogeographic distribution of resistance, as well as other relevant information about the nature of the predator/prey pairs in question. The discussion here centers on chemical arms races between venomous predators and resistant prey; that is, the focus remains only on animal/animal interactions, as there are no known cases of an animal venom used to subdue plant or prokaryote prey, or a plant that uses venom to dispatch prey species. Following the predator-specific sections is a concluding discussion of our current understanding of prey resistance to natural toxins, future directions for resistance research, and possible applications of resistance systems for practical and theoretical purposes.

Coevolution of Predator Venoms and Prey Resistance

When considering prey resistance, the underlying issue is whether a coevolutionary response to the selective pressure of predator venom exists within the system. Venoms, as derived trophic adaptations, are expected to experience selection pressure from mechanisms that allow prey species to evade predation. The appearance of resistance molecules in response to the derivation of new snake venom toxicities is expected to follow Dawkins and Krebs' (1979) model for an arms race between two taxa in an antagonistic coevolutionary relationship. A predator develops a chemical weapon (venom), which is used to subdue a prey species. As predators capitalize on susceptible individuals, the diversity of the prey population becomes limited to those individuals who are able to evade predation. These remaining individuals may persist because of phenomena like behavioral modifications, changes in microspatial distribution, or the appearance of a chemical mechanism that inhibits the toxic action of the predator's venom, namely, resistance. This resistance phenotype is expected to increase over time as the snake predator becomes increasingly incapable of incapacitating prey with the new resistance phenotype. Variations in predator and prey phenotypes are expected to follow each other through time in a frequency-dependent manner that creates new resistances to new toxicities and vice versa.

Several expectations follow from this scenario of the development and maintenance of resistance in prey. First, predator/prey pairs are expected to associate with each other for stable periods of time. By definition, predators and prey should respond in sequential and reciprocal manners as the opposing partner develops a new offensive or defensive strategy to the other. Van Valen (1973) described this stable reciprocity in his postulation of the Red Queen hypothesis. Much as the Red Queen in Lewis Carroll's *Through the Looking Glass* tells Alice that to stay in one place she must keep running, Van Valen hypothesized that for either predator or prey to "stay in one place" (i.e., persist through evolutionary time), they must continue to evolve. By extension, if one of the predator/prey pair was unable to continue to respond to a newly derived trait in the other partner, they would soon become

extinct, assuming intense predation pressures on the susceptible prey phenotype. Extant predator/prey pairs should demonstrate some balance between the relative abundance of resistant and susceptible individuals, keeping in mind that this balance may be skewed toward one partner or the other at any given time point.

In addition to stable reciprocity, the timeline of coevolutionary relationships is expected to develop over longer rather than shorter timescales. When investigating the frequency and mechanism of resistance, it may be that the newly evolved resistance or toxicity is at such low abundance that detection of this functionality is nearly impossible. In the real time of academic research, the turnover of enough generations of predator or prey species to produce a new functionality may be too slow for any given researcher to describe in a lifetime. Additionally, whether novel toxicity or resistance are diversifying or are being selected against may depend on the historic length of predator/prey associations. Sunagar and Moran (2015) compared the rate of diversification of a variety of toxin groups against the relative age of a number of venomous species' lineages. These authors proposed a "two-speed" mode of venom evolution, where more recent lineages of venomous predators, such as cone snails and venomous snakes, show increased diversifying selection, and older lineages appear to be under increased levels of purifying selection. The authors proposed that diversifying selection for venomous predators would be associated with prey base or niche expansion; however, it is possible that diversifying selection may allow for maintenance of a stable relationship with current prey species and simply throw frequency-dependent selection of a chemical arms race into another round of novel toxicity and resistance development. In any case, younger or older lineages are not fixed in a selective regime and may experience a switch from purifying to diversifying selection and vice versa. Thus, it appears that the age of the lineage in question may increase the likelihood that resistance is a prominent feature of prey populations or that the toxicity of the predator may have an advantage over prey defenses (such as in Holding et al. 2016), again making resistance more difficult to detect.

It is cogent to note that while a chemical arms race scenario is presently a "best guess factor" as the driving force for biochemical diversification of venoms over evolutionary time, numerous cases of prey-specific toxicities and venom resistances are documented in the literature, which lends support to a coevolutionary relationship between toxicity and resistance. In support of the chemical arms race scenario, research into the relationships between venomous snakes and their resistant prey will serve as a test case. Current information about a diversity of resistant prey is prefaced by a discussion of theoretical and methodological approaches to evaluating the importance of coevolutionary processes in the development of resistance.

Resistance to Snake Venoms

Natural resistance to predator venoms is best described in prey species of venomous snakes, particularly mammals. The impetus for this wealth of knowledge comes from the attempt by snake venom researchers to elucidate the merits of the

hypothesis that diet has served as a major selective pressure shaping snake venom composition. Over the past several decades, researchers have demonstrated that venom composition may vary across geographic space and ontogenetically (see Mackessy (2010)) and has been purported to vary with diet (e.g., Gibbs and Mackessy 2009; Sanz et al. 2006). The more recent championing of diet as a major driver for venom compositional change is born out of an institutional debate over the origin of venom, i.e., whether venom is the product of neutral or selective processes over evolutionary time.

Near the end of the twentieth century, the issue of the origin of snake venoms as the product of neutral or selective processes became a major theoretical divide between venomous snake biologists. Scientists such as Dietrich Mebs (2001) and Mahmood Sasa (1999) argued that because snakes delivered venom in such large quantities, many times more than was sufficient to incapacitate prey, venom must not have arisen from selective processes and was "overkill." Considering the discrepancy between the minimum amount of venom required for prey capture and the actual amount delivered, they argued that venom components were too metabolically costly to be used in such large quantities. Additionally, they noted that the individual components of venom were so toxic across a variety of possible prey species that there did not appear to be a selection for specific toxicities. To these authors, venom arose out of neutral evolutionary processes that allowed for the sequestration and concentration of modified somatic molecules into what we observe today as the components of snake venom.

This neutral view was quickly challenged by research showing that the notion of overkill was unlikely. Hayes et al. (2002) demonstrated that venomous snakes had control over the amount of venom released in striking a prey item. The amount of venom delivered was more than absolutely necessary to subdue prey items, but control over venom delivery indicated that there was a functional role for allowing large volumes to be expressed in snakebite envenomation. Saviola et al. (2013) demonstrated that, at least in venomous snakes from the family Viperidae (vipers, pit vipers, and other solenoglyph venomous snakes), a large bolus of venom was required in order to deliver a particular molecule in high enough concentration to allow the snakes to recover their envenomated prey item. Viperid snakes often use a sit-and-wait ambush strategy and strike prey as they cross the snake's path; prey that has fled the ambush site and succumbed to the effects of the venom is then recovered, often at some distance to the ambush site. The process of prey relocation may be challenging because prey may escape in any direction in three-dimensional space, and thus a relocator molecule is needed to track the envenomated prey item effectively. At this point, an arms race hypothesis was explored to explain the evolution of the complex phenotype of snake venom and associated delivery systems.

A number of prey species groups show resistance to snake venoms, and a wide variety of evidence helps to corroborate a chemical arms race scenario. Each species group will be treated separately, and data has been compiled on the prevalence and mechanism of resistance. Any study attempting to uncover coevolutionary relationships between species pairs faces the challenge of using extant and historical evidence to infer reciprocity across evolutionary time. A number of approaches are

often used and synthesized to confirm coevolution (Futuyma and Slatkin 1983). In the case of resistance/toxicity systems, the demonstration of resistance through standard toxicity assays is required. Anecdotal evidence for prey ability to avoid predation may not be explained by chemical resistance; resistance must be confirmed through direct challenges with physiologically and biologically relevant doses of venom. As novel phenotypes should appear in a single individual or small population of individuals and radiate out in the direction of gene flow, locality of both predator and prey must be taken into account. A record of the geographic distribution of populations with resistance or susceptibility may further allow for spatial correlation with the range of the venomous partner species. Thus, a biogeographic account of current resistance may be constructed. Longitudinal documentation of the biogeography of a particular resistance mechanism may offer some insight into the rate of change in the dynamics of resistance and toxicity for a given species pair. To date, it does not appear that this type of long view has been established for any system involving snakes, and even if one could be constructed, if reciprocal responses occur over evolutionary time, this may preclude any detection of active flux in the relationship between toxicity and resistance within the lifetime of a given researcher.

Following initial screening for resistance, mechanistic descriptions are often elucidated that demonstrate the direct ability of prey physiologies to negate the pathologic effects of venoms. As mentioned earlier, prey species are challenged by the (often) complex phenotype of predator venom, and their responses may range from a wholesale attempt to neutralize the diversity of toxins in a venom to mechanisms that attack a limited number of toxins. Finally, some attempt must be made to connect species pairs in evolutionary time and demonstrate stepwise evolutionary change. This correlation through time is the most difficult line of evidence to obtain, as current technologies limit these types of studies to phylogenetic comparisons between predator and prey species complexes (Filipiak et al. 2016; Page 2002; Suchan and Alvarez 2015). Correlation between the divergence of predator and prey clades would seem to indicate reciprocal evolutionary divergence; however, correlational analyses are limited in their ability to confirm causality between the coevolution of toxicity and resistance and speciation or divergence in predator and prey taxa. It is also possible that some common biotic or abiotic pressure, unrelated to potential coevolutionary scenarios, caused cladogenesis in both predator and prey species, and resistance is secondarily derived.

Resistance to Snake α-Neurotoxins

A resistance mechanism that has been confirmed across a diversity of mammalian predators and prey is the ability to tolerate snake α-neurotoxins, acetylcholine receptor (AChR) agonists. Ovadia and Kochva (1977) demonstrated that mongoose sera challenged with venoms from snakes in the family Elapidae (cobras, kraits, and other opisthoglyphous snakes) was able to neutralize the effects of the venom.

Later research uncovered that this resistance to elapid venoms is directed against α-neurotoxins that make up a significant portion of the total venom protein. Barchan et al. (1992) sequenced the mongoose AChR and detected a number of non-synonymous mutations in the ligand binding site of the AChR. Hypothesized structures for these mutations indicate a confirmation change in the ligand binding site that prevents α-neurotoxins from binding while still allowing acetylcholine (ACh) to bind its receptor. Later work (Asher et al. 1998) further demonstrated that the mongoose's resistant AChR prevented α-neurotoxins from binding while still allowing ACh to bind with higher affinity than non-resistant type AChR found in rats. This elevated binding affinity indicated that mongoose AChR was able to prevent complete binding of α-neurotoxins while allowing ACh to bind with little steric or concentration-dependent competitive hindrance from α-neurotoxins that had inundated synaptic junctions. A slight conformational change was sufficient to create near complete resistance to α-neurotoxins.

In addition to mongooses, similar conformational changes in acetylcholine receptors have been documented in the Chinese cobra (*Naja atra*), the Javelin sand boa (*Eryx jaculus*), the dice snake (*Natrix tessellata*), and also in the European hedgehog (*Erinaceus europaeus*) (Barchan et al. 1992; Neumann et al. 1989). Resistance in *N. atra* is most likely protection against auto-envenomation; however, it is possible that this resistance may allow evasion from cannibalism or predation by other sympatric elapid snakes. The example of *E. europaeus* provides an additional mammalian example of resistance to α-neurotoxins, but perhaps the most intriguing example of resistance is the case of the three non-venomous snakes. Considering the ongoing debate among snake venom toxinologists about the ultimate origin of snake venom proteins and the delivery apparatus (e.g., Fry et al. 2012), the appearance of α-neurotoxin resistance across more basal snake taxa begs the question of whether resistance is intrinsic to snake physiology or has appeared independently several times throughout the radiation of the snakes. In any case, a better understanding of the molecular origin of snake resistance to snake venoms could indicate a coevolutionary predator-prey situation if the hypothesis that resistant, non-venomous snakes were once or are currently preyed upon by venomous snakes is supported.

Resistance in Woodrats (Genus *Neotoma*)

As a follow-up study to anecdotal evidence of resistance in Southern Plains woodrats (*Neotoma micropus*), Perez et al. (1978) challenged woodrats with venom from the western diamondback rattlesnake (*Crotalus atrox*), showing that these rodents had greatly elevated tolerance to the venom compared to a laboratory mouse control. Perez et al. (1979) further showed that this resistance mechanism was able to significantly decrease the hemorrhagic effects of *C. atrox* venom for *N. micropus*. De Wit (1982) screened a second *Neotoma* species, the eastern woodrat (*Neotoma floridana*), with the venom from Osage copperhead (*Agkistrodon contortrix*

phaeogaster) and detected a similar resistance to hemorrhagic toxins. It appeared that venom resistance was shared across the genus. Using electron microscopy, Huang and Perez (1982) further showed that *N. micropus* suffered little hemorrhage or muscle damage following envenomation. Some mitochondrial and myofibril damage were detected, but it appeared that resistance also prevented myotoxic pathologies, especially in comparison to laboratory mouse controls. A candidate antihemorrhagic resistance molecule was purified and partially described by Garcia and Perez (1984). This single, non-enzymatic resistance molecule was able to bind and neutralize *C. atrox* toxins. Binding was shown to be non-polyvalent, and the authors concluded that this candidate molecule was not an immunoglobulin. Unfortunately, it does not appear that further descriptive work has been completed on this resistance molecule, and no biogeographic or further phylogenetic information is available regarding the distribution and prevalence of this resistance mechanism in *Neotoma*.

Resistance of Ground Squirrels (Genus, *Otospermophilus*) to Snake Venom Metalloproteases

Another well-described example of snake venom resistance are endogenous snake venom metalloprotease inhibitors (SVMPIs), best documented in a number of squirrel species in the genus *Otospermophilus* (formerly *Spermophilus*). Biardi and Coss (2011) showed that rock squirrel (*Otospermophilus variegatus*) serum was able to neutralize the pathological effects of venom from two species of rattlesnake, the western diamondback rattlesnake (*Crotalus atrox*) and prairie rattlesnake (*Crotalus viridis viridis*), which were sympatric to assayed squirrel populations. Challenges with venom from an allopatric species of rattlesnake, the northern Pacific rattlesnake (*Crotalus oreganus oreganus*), were not successfully neutralized. Interestingly, the venom used in these experiments was commercially purchased; however, even without a confirmation of matching locality between predator and prey samples tested, there still appeared to be an inhibitory effect against individuals from a sympatric predator species. In the same year, another team (Biardi et al. 2011) published a description of an SVMPI isolated from *O. beecheyi* serum. This molecule was able to prevent tissue damage and hemorrhage normally expected from envenomation by the sympatric *C. o. oreganus*. Further, resistance was positively correlated with the proximity of rattlesnake population to resistant *O. beecheyi*; that is, resistance was ineffective against distant populations of *C. o. oreganus*, indicating that resistance is geographically localized and requires predation (or at least offensive) pressure from the colocalized rattlesnake population to select for resistance. The authors recognized that while other mammals do not have similar SVMPIs that serve as resistance molecules, there appears to be convergence of defenses against hemorrhagic toxins, a hallmark of many viperid snake venoms. Future work in mammalian resistance to viperid venoms will confirm or reject convergence to defenses against hemorrhagic toxin classes of snake predators.

Resistance to Snake Venoms in the Opossums (Family Didelphidae)

A final group of prey items with described resistance to venomous snake predators are the opossums (Mammalia: Didelphidae). Jansa and Voss (2011) reported an increased number of non-synonymous changes in gene sequences of a hemostatic protein, von Willebrand factor (vWF), in opossums known to exploit venomous snakes as prey items. These researchers found that these non-synonymous changes are associated with binding sites for C-type lectin-like proteins found in some viperid snake venoms; changes to these regions were inferred to decrease binding affinity with these toxins. These data do not indicate that opossums preyed upon by venomous snakes have similar resistance, but later work (Voss 2013) found that a number of opossum species could be confirmed as venomous snake prey and that their relationships to known, resistant species of opossums make it plausible that they would also likely show changes to vWF. However, beyond these types of phylogenetic correlations, evidence for resistance against venom challenges is not available, and physiological data would be required to verify that resistance to C-type lectin-like proteins is sufficient to allow for evasion from predation by venomous snakes.

Correlational Evidence for Resistance/Toxicity Coevolution in Venomous Snakes

The extent of information regarding resistance to snake venoms varies depending on the species group of interest and may include as little as an initial confirmation of resistance to a full description of the resistance mechanism. In relatively few cases, functional information can be paired with evolutionary analyses to test the underlying assumptions of a chemical arms race. Barlow et al. (2009) investigated a potential coevolutionary relationship between venom specificity toward scorpion prey in four species groups of the genus *Echis* (saw-scaled vipers). They used a Bayesian inference method to plot a phylogeny of these four groups and compared the relative amounts of scorpion versus rodent prey found in the stomach contents of museum specimens, as well as toxicity assays (LD_{50}) toward scorpions (*Scorpio maurus*), to species relationships. Venoms of species groups with the highest amounts of scorpions in their diet were the most toxic against scorpion prey, while the *E. coloratus* group, rodent specialists, showed the lowest toxicity. Relative abundance of a particular type of prey scaled with the relative toxicity of the venom; for example, the *E. ocellatus* group had an intermediate amount of dietary scorpions and showed an intermediate toxicity toward live scorpion prey. The implication of this increased toxicity toward preferred prey group was that *Echis* venom has undergone selection favoring increased toxicity toward a preferred prey type. While Barlow et al. (2009) did not test for scorpion resistance, the demonstration of prey specificity that follows the best resolution of *Echis* phylogenetic relationships indicated a positive selective pressure for enhanced toxicity, perhaps driven by prior prey resistance mechanisms. For example, a common ancestor to

Echis may have retained toxicity toward scorpions, while sympatric Rodentia developed resistance, to the point that only *Echis* phenotypes that could shift to non-rodent prey were able to persist. Secondary diversification of the venom toxins may have restored high toxicity toward rodent prey, favoring a shift in those lineages to specializing on rodents. The availability of non-scorpion taxa, preference toward these taxa (how often they attempt to predate), and the relative resistance or susceptibility of these taxa would be needed to corroborate reciprocal selectivity of venom and resistance.

In the case of opossums, antihemorrhagic toxicity has been correlated with phylogenetic comparisons of predator and prey species. Voss and Jansa (2012) compared South American opossums and vipers, revealing that species of opossums that were too large as adults to be ingested by vipers showed no resistance to venom. Nonresistance in larger prey taxa was interpreted as the result of non-predation that venomous snakes had no behavioral inclination to attempt predating these overly large meals and thus no selective pressure to develop resistance was present. Verifying the assumption of reciprocity between predator and prey, resistance may arise or be maintained only in prey lineages that are likely targets of venomous snake predators.

Natural Resistance in Prey of Other Venomous Taxa

Presently, little information is available regarding the appearance or mechanisms of resistance in prey species of cone snails, insects, helodermatid lizards, cnidarians, centipedes, shrews, scorpions, arachnids, and anemones. The sporadic and sometimes tangential evidence that exists for resistance against a number of these venomous predators will now be discussed. Literature searches for documented cases of resistance in prey species of insects were unproductive, but protective immune reactions in non-prey species may indicate a set of mechanisms that provides resistance for prey. Metz et al. (2006) described the ability of mast cells in inbred laboratory mice to confer protection against hypothermia and death associated with envenomation by the European honeybee (*Apis mellifera*). Palm and Medzhitov (2013) later demonstrated that whole honeybee venom and the isolated pore-forming toxin, melittin, was able to induce inflammatory pathways in in vitro and in vivo experiments. The honeybee does not use its venom for prey capture; however, it may be that resistance to venoms of bee relatives in the order Hymenoptera, such as predatory wasps, rely on the escalation of similar immune and allergic responses to evade predation.

Immune responses conferring resistance to envenomation have been documented for some arachnids. Schenone et al. (1970) induced resistance to challenge doses of venom in laboratory rabbits through repeated sublethal doses of venom from the Chilean recluse spider (*Loxosceles laeta*). A ramping of immune response to venom dosing was detected by observing the increasing presence of antibodies in rabbit serum across the dosing period. Similarly, Njau et al. (1986) induced resistance to paralysis in laboratory rabbits through repeated sublethal infestations of red-legged

ticks (*Rhipicephalus evertsi evertsi*). Later, Reck et al. (2009) used serum from tick-infested cattle to confer protection again the anti-hemostatic properties of tick saliva in in vitro and in vivo assays. While defenses to parasitism by tick species do not fit with a definition of prey resistance to venom, the apparent excitation of the immune system in cattle speaks to a convergent mechanism by which arachnid venoms may be neutralized. As arachnid toxins are quite diverse, hypothesizing a general convergent mechanism may be too simplistic, but it stands to reason that in the absence of other candidate resistance mechanisms to explore, immune responses to arachnid venoms are plausibly productive.

Other than immune-based resistance to arachnid venoms, research into the application of arachnid toxins as insecticides has revealed another possibly fruitful avenue of study regarding prey resistance to arachnid venoms: the prevention of toxin binding to nervous cell receptors by structural interference. Bende et al. (2014) identified two residues in a particular region of American cockroach (*Periplaneta americana*) voltage-gated sodium channels that conferred resistance against β-Diguetoxin-DC1a from the desert bush spider (*Diguetia canities*). These researchers were attempting to discover novel targets for insecticide development and in the process uncovered the mechanism whereby some insects may avoid envenomation by desert bush spiders. Differential toxicity to prey nervous tissue has been identified for other spider predators. For example, Liu et al. (2016) documented the ability of *Araneus ventricosus* venom to block cockroach, but not mouse, voltage-gated sodium channels, suggesting the binding mechanism causes lethal effects in insects while inactive toward vertebrates. In both cases, the experiments were motivated by the development of insecticides that are insect-specific; however, these lines of inquiry reveal possible candidate resistant prey species.

Another group with preliminary evidence for resistance in prey is the sea anemones (phylum, Cnidaria; class, Anthozoa). Some species of this group capitalize on prey species that are powerful enough to escape the grasp of an anemone, such as teleost fishes, or have durable defenses to infiltrate, such as mollusks, which necessitate the use of venom for prey capture (Frazão et al. 2012). While direct evidence of the development of resistance in putative prey species is not available, there are a number of studies that indicate two mechanisms that confer resistance to mutualistic anemone fishes (genera *Amphiprion* and *Premnas*) and crustaceans (representatives from several genera; Mebs 2009). First, mutualistic partners may develop or acquire a mucus coat that neutralizes defensive compounds on the surface of the anemone, or else allow the partner to associate closely with the anemone without eliciting the firing of venom-delivering stinging cells, nematocysts (Frazão et al. 2012). A second line of defense in mutualistic partners of sea anemones are internal defenses that allow the partners to neutralize venom toxins, should the nematocysts fire. Mucus coat defenses appear to be the main defense for mutualistic crustaceans (Mebs 2009), and mutualistic anemone fish appear to use combinations of both strategies. Mebs (1994) tested three mutualist *Amphiprion* anemone fish species against the venom of four sea anemone species, finding limited endogenous resistance in cohabitating fish species. In some cases, the mutualist fish was not resistant to the venom of its own host anemone. Together, these trends indicated that

the development of a protective mucus coat was the main defense against host venom for anemone fish and that resistance may or may not be necessary for successful mutualistic relationships. A survey by Nedosyko et al. (2014) of the number of associations between all 26 species of mutualist anemone fishes and all ten species of host anemones indicated that anemones with the least and most toxic venoms were inhabited by the fewest numbers of mutualist species. Intermediate toxicity was associated with the greatest diversity of mutualist species, and these authors concluded that there must be a trade-off in the amount of protection versus the amount of risk for potential mutualist species. For putative prey species of anemones, differential toxicity across anemones may reflect a variegated landscape of selective pressures that could lead to the development or refinement of resistance mechanisms. However, no evidence of resistance in prey species is currently available. One mechanism of resistance that may be of interest for future investigation is changes in the architecture of ion channels of sea anemone prey species. Gasparini et al. (2004) compared the previously documented ability of scorpion and sea anemone venoms to block voltage-gated potassium channels, indicating convergence on the same toxic mechanism, i.e., binding a specific portion of the pore complex to prevent the passage of current through these channels. Thus, candidate resistance mechanisms to sea anemone venoms may arise as the result of non-synonymous changes to exposed surfaces of ion channels that reduce the ability of toxins to bind and block physiological currents. This kind of change has given rise to the tetrodotoxin resistance seen in red-sided garter snakes (*Thamnophis sirtalis*), allowing the predator to capitalize on otherwise deadly prey (Feldman et al. 2012; McGlothlin et al. 2014).

Finally, resistance to scorpion venoms has been documented, but further investigations of the mechanisms or biogeography of resistance have yet to appear in the literature. Israeli-Zindel et al. (1973) derived LD_{50} values for venom of the yellow scorpion (*Leiurus quinquestriatus*) toward seven species of beetles and a strain of laboratory mouse. They found a wide range of susceptibility and resistance and demonstrated that several beetle species tested had several orders of magnitude greater tolerance to the venom than the laboratory mouse. When the hemolymph of the most resistant beetle was analyzed 24 h following envenomation, detectable venom concentration had dropped to 40% of the original level. A further assay testing the specificity of resistance revealed that an enzyme-deactivating mechanism confers resistance to this beetle species. However, beyond this early study, few have tested the ability of plausible prey species to defend against scorpion envenomation, and most studies focus on species that are unlikely prey of scorpions, such as rodent predators of scorpion (Rowe and Rowe 2008). As in other non-snake predators mentioned above, there is evidence from tests in model organisms that immune responses may be likely resistance mechanisms for some prey items (see Akahoshi et al. 2011; Kamon and Shulov 1965), but it remains to be seen whether these are mechanisms present in scorpion prey species. Collectively, the literature presents a range of possible resistance mechanisms to venomous predators, and future research may confirm the presence of resistance in prey species.

Explanations of a Limited Literature on Natural Resistance

In general, it appears that natural resistance to predator toxins should appear, yet available information is limited. Reaffirming the likelihood that predation pressures, particularly the trophic adaptation of venom, should drive coevolutionary development of resistance, several explanations for a lack of information on resistance emerge. First, a dearth of reported resistance may result from variable and insufficient research effort: the simplest explanation would be that little or no effort has been made to screen candidate resistant prey. Even in the most well-described resistance systems, resistance to venomous snakes, mammalian resistance dominates the literature, despite abundant natural history accounts of venomous snakes consuming nonmammalian prey (but see Mackessy and Saviola (2016)). Second, while some effort may have been made to investigate predator/prey interactions, the documentation of local specificity in some of the prey resistance systems discussed suggests that analyses may not detect resistance because of mismatches between the localities of predator and prey that are tested. The maintenance of resistance in a population of prey species may be dependent on the presence of a particular venom profile that in turn is delimited by the overlapping ranges of local populations of predator and prey. Thus, assaying for resistance using a venom from outside of assayed individuals' local area may lead to the false conclusion that resistance is not present in a prey species or population. Third, beyond mismatching of predator/prey populations, small sample sizes also may allow resistant prey to be overlooked. Under a Red Queen dynamic, the frequency of resistance is expected to cycle through periodic minima. Low-frequency resistance phenotypes would be increasingly harder to detect by random sampling. All in all, future investigations in these least described predator/prey systems and continuing investigations in known resistance systems must consider that limitations in research design and effort may not capture the evolutionary processes driving reciprocal flux between resistance and toxicity.

Another explanation for limited information on prey resistance is the possibility that these predators do not exert enough predation pressure to cause selection for prey resistance. Simply, prey resistance may not exist, despite the logic of coevolution under a chemical arms race hypothesis, because venomous species are not significant predators. If predators move from specialist to more generalist diets over time, selection of novel toxicities may be favored, and therefore reciprocal resistance may not appear. Initial development of toxicity against a limited number of prey species may allow predators to capitalize later on a wider range of related prey species with similar physiologies. With a wider prey base, predators would be able to take advantage of other food sources in the event that resistance does appear in some prey individuals. Therefore, if selective pressure from venomous predators is negligible, and the appearance of resistance alleles in a population only happens as a result of random mutation, the fixation of prey resistance in the population is unlikely, because these rare resistance alleles risk early extinction due to their low abundance. Finally, over time, overcoming the toxic action of venom by prey may

prove insurmountable, and our present-day analysis would detect venom toxicity to a variety of locally available prey, but no or extremely small numbers of resistance mechanisms in prey. The present discussion only considers chemical resistance to predators' venoms, but other strategies may evolve in response to the selective pressure of venom toxicity. Behavioral modifications, and/or reproductive strategies that allow further generations of prey to persist in an area, may subvert the predation pressures of venomous animals and bypass chemically based coevolutionary processes. For example, in one of the better described toxicity/resistance dyads (between Pacific rattlesnakes and ground squirrels), several behaviors that prevent predation are documented. Certain populations of squirrels are known to tail flag to signal their awareness of a nearby predator, resulting in the retreat of the approaching rattlesnake (Putman and Clark 2014); others bombard approaching rattlesnakes with substrate to motivate predator retreat (Goldthwaite et al. 1990), and some rub themselves against shed skins of local rattlesnakes to mask individual scent and evade chemosensory detection (Clucas et al. 2008). While these populations may also have chemical defenses against predator venoms, behavioral modifications that disrupt predatory episodes exist as well, demonstrating that other prey species may not require physiological resistance mechanisms if behavioral modifications are sufficient to elude detection and/or envenomation.

Conclusion and Future Directions

The diversity and efficacy of prey resistance appears to be shaped by the selective pressure of predator toxicity as predicted by chemical arms race hypotheses. However, the fact that only a handful of well-described resistance systems exist in the literature demonstrates the need for further investigations into the diversity and extent of prey resistance. Future directions in the study of natural resistance to venoms must include screens for resistant prey species, using in vitro or in vivo assays to identify the capacity of prey species to avoid the normally pathological consequences of envenomation. Development of a well-supported alternative to LD_{50} determinations is crucial to reduce the number of native prey animals needed to demonstrate resistance and increase throughput, but at present there is no sufficient model to replace whole animal toxicity tests, particularly for unknown systems. Special attention should be paid to the interaction of local populations of predators and prey versus the effects of predator venoms on nonlocal populations of (possible) prey. Further, the prevalence of resistance mechanisms that appear specific to local predators indicates that the development and propagation of resistance genotypes could be modeled to predict or detect the appearance of new resistance mechanisms or to track the spread of resistance mechanisms through prey populations across large landscapes that connect multiple populations. The detection of local resistance also may indicate that current information about the relative abundance of resistance in a given prey species is underestimated; multiple pairwise comparisons between local predator and prey populations would be required across a significant portion of their sympatric range to document resistance or susceptibility unequivocally.

Understanding that evolutionary processes are adequate but not necessarily ideal, reciprocal stepwise modifications to either toxicity or resistance mechanisms are expected to be the norm in coevolutionary systems, rather than wholesale changes to composition. The recent use of genome/transcriptome/proteome comparisons (i.e., Cardoso et al. 2010; Gibbs et al. 2009) could shed light on underlying trends in molecular evolution: how often do resistance genotypes change, how often do novel genotypes appear, and what resistance mechanisms are likely to experience the strongest selection?

Beyond research opportunities focusing on the evolutionary history and development of prey resistance, a better understanding of resistance mechanisms may provide a source for future biomedical innovation. Currently, clinical treatment, both medical and veterinary, of envenomation by venomous species commonly relies on the use of antivenom therapeutics and complementary treatment regimens to combat systemic pathologies such as hypofibrinogenemia, thrombocytopenia, myotoxicity, neurotoxicity, and many other symptoms (Chippaux and Goyffon 1998; Diaz 2004; Rhoads 2007). The incidence of envenomation by spiders, scorpions, and snakes are of particular concern considering their common occurrence, dramatic impacts to global health, and significant financial impacts to health systems. In an attempt to improve treatment, the World Health Organization (WHO 2007) deemed envenomation by snakes and scorpions to be a neglected public health issue and has suggested strategies to develop better antivenom therapeutics. While improvement of existing antivenom therapeutics promises to increase the efficacy of envenomation treatment, the addition of venom resistance molecules to treatment protocols may further improve clinical outcomes. Resistance molecule therapeutics are not intended to replace antivenom therapies, but instead work synergistically with existing treatment protocols to combat venom toxicities. As proof of concept, two classes of anti-snake venom compounds derived from resistant prey species have been cited as promising candidates for drug discovery. Thwin et al. (2010) provide a summative review of a number of these molecules, including a group of phospholipase A_2 inhibitors (PLIs) derived from venomous snake blood sera (Viperidae, Elapidae). The biological roles of these molecules is to prevent complications from auto-envenomation or envenomation by other sympatric (intra- and interspecific) venomous snakes. Hypothetically, clinicians could administer the appropriate antivenom to combat broad spectrum effects of envenomation and additionally employ a derived PLI in cases where patients present with envenomations from snakes with PLA_2-rich venoms. Treatment schedules that incorporate such molecules could be better tailored to individual patient needs to improve the efficacy of medical intervention and patient health outcomes.

In addition to PLIs, another promising class of resistance molecules for drug development are snake venom metalloprotease inhibitors (SVMPIs). As mentioned earlier, SVMPIs have been isolated from a wide range of mammalian prey species of snake predators. Especially in the Americas, SVMPIs promise an excellent addition to combat the hematologic pathologies experienced in a large number of snakebite envenomations (owing to a higher proportion of venomous taxa with snake venom metalloprotease-rich venoms; Mackessy 2010). Metalloproteases have been

described as "gateway toxins" (Biardi et al. 2011) because they break down structural elements within tissues, potentially increasing the rate that other toxic components of the venom may infiltrate and access the bloodstream. Biardi et al. (2011) postulated that the therapeutic use of an SVMPI would limit access of venom components by destroying the ability of the venom to spread from the envenomation site. The biochemical functions of metalloproteases (hemorrhage, tissue destruction) would be blocked, and spread of venom would be attenuated, and the hope is that this temporary neutralization of one part of the venom and subsequent sequestration of other toxins would allow antivenom therapeutics time to propagate to and neutralize the locally envenomated tissue. In short, resistance molecules such as PLIs and SVMPIs are expected to shorten treatment regimens by increasing immediate efficacy of antivenom therapeutics.

In conclusion, our understanding of the prevalence and mechanisms of prey resistance to natural toxins remains limited to a small number of predator/prey systems. However, the prediction that prey species in tightly coupled predator/prey relationships should develop reciprocal chemical arms against predator toxins motivates a continued effort to discover and describe resistance. Future studies should focus on assessing not only the mechanistic nature of resistance but also the demography of resistance in natural populations of prey. Dedication to interdisciplinary approaches that couple molecular and ecological information will exponentially increase what we understand of the interactions between venomous predators and their resistant prey.

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Part II

Toxin Evolution in Major Venomous Groups

A Critique of the Toxicoferan Hypothesis

4

Adam D. Hargreaves, Abigail S. Tucker, and John F. Mulley

Abstract

Historically, venom was believed to have evolved twice independently in squamate reptiles, once in the advanced snakes and once in venomous lizards. The presence of putative toxin proteins in the saliva of species usually regarded as non-venomous, and the expression of venom gene homologs in their salivary glands, led to the hypothesis that venom evolved a single time in reptiles. As the single, early origin of venom is synonymous with the Toxicofera clade (Serpentes, Anguimorpha and Iguania), it will subsequently be referred to as the Toxicofera hypothesis. This hypothesis has proved to be remarkably pervasive for almost a decade, but has until recently never been tested. Here, evidence used in support of the Toxicofera hypothesis is reviewed and critically evaluated. Taking into account both new and old data, it appears that this hypothesis is unsupported, and should be subject to further scrutiny and discussion. Finally, the implications of the rejection of the Toxicofera hypothesis are discussed, with respect to the knowledge of venom evolution in the Reptilia and also the practical implications of this knowledge.

Keywords

Toxicofera • Reptiles • Venom • Oral glands • Venom glands

A.D. Hargreaves (✉)
Department of Zoology, University of Oxford, Oxford, UK
e-mail: adam.hargreaves@zoo.ox.ac.uk

A.S. Tucker
Department of Craniofacial Development and Stem Cell Biology, King's College London, Guy's Hospital, London, UK
e-mail: abigail.tucker@kcl.ac.uk

J.F. Mulley
School of Biological Sciences, Bangor University, Bangor, Gwynedd, UK
e-mail: j.mulley@bangor.ac.uk

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_4

Contents

Introduction

Venomous reptiles have long been the source of fear and fascination in roughly equal measure, not least because of the extensive annual global mortality and morbidity caused by reptile envenomation, particularly in the developing world (Kasturiratne et al. 2008; Harrison et al. 2009). Research effort has traditionally focused on the characterisation of venom toxins and the development of treatments to counteract their clinical effects, and so species considered to be medically important have received the most attention (for example, the saw scaled vipers (Wagstaff and Harrison 2006; Wagstaff et al. 2009; Casewell et al. 2009)). As a consequence, the full evolutionary history of venom in the Reptilia has remained unknown, and to this day poses unanswered questions, including fundamental topics such as the origin of venom toxins, what constitutes venom and a venomous animal and even the timing of the evolution of venom itself.

Hypotheses concerning the evolution of venom within reptiles have undergone dramatic revision within the last decade, and are currently in a state of flux. Historically, venom within reptiles was believed to have evolved twice independently: once in the Caenophidia (advanced snakes) and once in the Helodermatid lizards (Gila monsters and beaded lizards) (Kochva 1978; Pough et al. 2004) (Fig. 1). This belief was mainly due to the distant phylogenetic relatedness of these animals and clear differences in the morphology of their respective venom delivery systems (Kochva 1978; Saintgirons 1988). A more recent, alternative hypothesis (which we refer to as the "Toxicofera hypothesis") has become widely accepted within (and seemingly far beyond) the toxinological community. The Toxicofera is a clade of squamate reptiles comprising Iguania, Anguimorpha and Serpentes, whose name refers to the presence of venom within at least some members of these groups (Vidal and Hedges 2005). Phylogenetic analysis utilising nine nuclear genes (*α-enolase*, *amelogenin*, *c-mos*, *hoxa13*, *jun*, *mafb*, *rag1*, *rag2* and *r35*) found this clade to be strongly supported (Vidal and Hedges 2005), and this support has been reproduced in subsequent studies (e.g., Pyron et al. 2013). However, phylogenetic relationships within the Toxicofera are unresolved based on nuclear data, although the use of

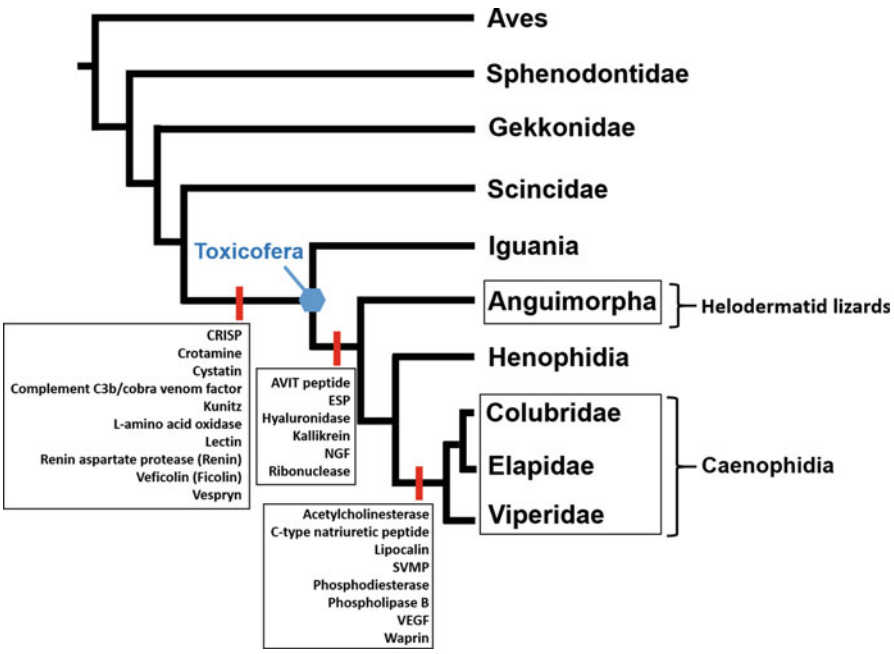

Fig. 1 Simplified Reptile cladogram. The phylogenetic position of venomous Helodermatid lizards and the Caenophidia (advanced snakes) are indicated. The phylogenetic position of the proposed venomous Toxicoferan ancestor also indicated along with the three proposed punctuated toxin gene recruitment events. Proposed recruited toxin gene families are also shown

SINEs (short interspersed nuclear elements) has suggested a clustering of snakes with anguimorph lizards (Piskurek et al. 2006) which is also supported by a more recent analysis (Hsiang et al. 2015).

The majority of the roughly 2,500 species of snake are classified within the Caenophidia, a sub-order containing four major lineages: Atractaspidinae; Viperidae (vipers, pit vipers); Elapidae (such as cobras and mambas) and Colubridae (a polyphyletic group which is constantly undergoing taxonomic revision) (Quijada-Mascarenas and Wüster 2009). Approximately 600 species, all belonging to the former three lineages, were traditionally considered to be venomous in that they possessed venom glands surrounded by compressor muscles, tubular fangs at the front of the mouth and are of medical significance to humans (although medical significance to humans is obviously a poor criterion on which to base classification of toxicity). Whilst some members of the Colubridae are opisthoglyphous (rear fanged), they do not generally pose a threat to humans and have historically not been considered to be venomous.

Evidence for a wider use of venom within advanced snakes was initially based on proteomic analysis of the saliva of the radiated rat snake (*Coelognathus radiatus*), a snake reliant on constriction for prey capture, where a post-synaptic neurotoxin belonging to the three finger toxin (3Ftx) family was discovered (Fry et al. 2003a).

This protein was found to possess the typical ten conserved cysteine residues of elapid 3Ftxs and when functionally tested led to antagonism of nicotinic acetylcholine receptors. This protein was therefore considered to be structurally and functionally homologous to the elapid three finger toxins (Fry et al. 2003a) and phylogenetic analysis showed strong support for the nesting of the rat snake 3Ftx within a clade of previously categorised 3Ftxs (Fry et al. 2003b). On the basis of these results it was suggested that three finger toxins were recruited into the venom repertoire prior to the divergence of the Elapidae and Colubridae (Fry et al. 2003a). Indeed, the analysis of other colubrid "venoms" (Mackessy 2002) added further support that the use of venom in the advanced snakes pre-dated their radiation in the Cenozoic era (Vidal and Hedges 2002). More interestingly, the presence of putative toxin proteins in the saliva of lizard species usually regarded as non-venomous (such as the lace monitor, *Varanus varius*), and the expression of venom gene homologs in their salivary glands, led to the proposed hypothesis that venom evolved a single time in squamate reptiles approximately 170 Mya (Fry et al. 2006), and not twice independently as had been previously believed (Pough et al. 2004; Kardong et al. 2009).

The timing of venom gene recruitment events within reptiles has undergone significant modification over the course of subsequent Toxicofera-related studies, with further sampling leading to the detection of an increased number of putative venom genes in a diverse collection of species (Fry et al. 2009, 2010, 2012a, 2013). These findings suggest an increasingly complex view of venom gene recruitment throughout the evolution of the Toxicofera, which has even extended to include the Komodo dragon (*Varanus komodoensis*). This species was previously considered to be reliant on oral bacteria (e.g., see Bull et al. 2010) to induce septicaemia in prey items, but is now considered to be venomous (Fry et al. 2009).

Here, the foundation and expansion of the Toxicofera hypothesis and the proposed single, early evolution of venom in reptiles are discussed and examined. The assumptions and key shortcomings of the evidence used in support of this hypothesis are reviewed, taking into account more recent findings and novel interpretations.

The Toxicofera Hypothesis

The first proposal of the single, early origin of venom in reptiles occurred in 2006 based upon the detection of genes homologous to those previously identified in the venom glands of venomous snakes expressed in the mandibular salivary glands of four Varanid lizards (*Varanus acanthurus*, *V. mitchelli*, *V. panoptes rubidus* and *V. varius*) and a single Iguanian (*Pogona barbata*) (Fry et al. 2006). Phylogenetic analysis demonstrated that nine toxin families were shared between these non-venomous lizards and advanced snakes: AVIT peptide; B natriuretic peptide; cysteine-rich secretory protein (CRISP); cobra venom factor (which is in fact complement component C3 (Alper and Balavitch 1976)); crotamine; cystatin; kallikrein; nerve growth factor and vespryn. Additionally, a type III phospholipase A_2 (PLA_2) was detected in the mandibular salivary glands of *Varanus varius* (Fry et al. 2006).

Subsequent Toxicofera-related studies mainly focused on the inclusion of additional lizard species (Fry et al. 2009, 2010, 2013). A more recent study sequenced cDNA derived from the oral glands of Iguanian lizards and Henophidian snakes using 454 pyrosequencing (Fry et al. 2013). The detection of apparent homologs of several Toxicoferan genes in these species led to a number of proposed gene recruitment timing events being shifted even earlier in Toxicoferan evolution, in some cases by up to 112 million years, and the adoption of a punctuated evolutionary history of toxin recruitment. In this scenario, three rounds of toxin gene recruitment have been proposed to have occurred in the Toxicofera: up to ten at the base of the Toxicofera (cysteine-rich secretory protein (CRISP), crotamine, cystatin, cobra venom factor, kunitz, L-amino acid oxidase, lectin, renin aspartic protease, veficolin, vespryn), six in the ancestor of Serpentes and Anguimorpha (AVIT peptide, epididymal secretory protein, hyaluronidase, kallikrein, nerve growth factor, ribonuclease) and eight (acetylcholinesterase, lipocalin, C-type natriuretic peptide, snake venom metalloproteinase, phosphodiesterase, phospholipase B, vascular endothelial growth factor, waprin) in the common ancestor of the Caenophidia (Fry et al. 2013) (Fig. 1).

The Toxicofera hypothesis proposes the existence of an early venomous squamate that would have possessed toxin-secreting glands on both the upper (maxillary) and lower (mandibular) jaw (Fry et al. 2006). The venom delivery systems in advanced snakes and lizards are therefore homologous but morphologically distinct derivatives of this primitive system, with snakes retaining the maxillary venom glands and venomous lizards maintaining the mandibular glands (Fry et al. 2006), with the opposing glands being secondarily lost by each lineage. It has been proposed that members of the Iguania (such as the green anole lizard, *Anolis carolinensis*) diverged whilst this venom system was in an incipient stage, and so lack any form of specialised toxin secreting glands. Furthermore, snakes which use alternative prey capture methods such as constriction are proposed to have secondarily lost venomous function (Fry et al. 2006).

Alongside the conserved shared expression of homologous genes, the conserved structure of homologous proteins has also been used to support the Toxicofera hypothesis, namely the conserved cysteine structure and functional residues (Fry et al. 2006).

Several Toxicofera-related studies have also included functional tests on the mandibular oral secretions of two varanid species, *Varanus komodoensis* and *V. varius* (Fry et al. 2006, 2009). Samples of crude oral secretion and purified natriuretic peptide were injected intravenously into anaesthetised male rats, which resulted in a drop in mean arterial pressure (MAP). Platelet aggregometry was also carried out using purified type III PLA_2 from *V. varius* which showed inhibition of platelet aggregation when tested on human blood samples.

Shortcomings of the Toxicofera Hypothesis

The Toxicofera hypothesis assumes that shared expression of a gene between what were previously considered non-venomous species and more derived venomous species implies shared toxicity (or at least a shared venomous ancestry) (Fry et al.

2006). It is of course plausible that homologous tissues (e.g., the venom gland and other oral glands) within related species will express similar complements of genes, and therefore presence alone does not provide any evidence of toxicity. Indeed, many of the proposed toxins which have been used to support the Toxicofera have never been functionally characterised. Moreover, the products of several of these genes have never been suggested to be toxic (for example cystatin type E/M (Ritonja et al. 1987)) or have been shown to not be toxic, even up to high doses, through functional tests (for example, acetylcholinesterase (Cousin et al. 1996)). Therefore these genes have been used to support shared ancestral toxicity, without actually functioning as toxins. Additionally, it now seems certain that many of the proposed shared venom toxins within the Toxicofera actually result from the confusion of orthologs and paralogs, where non-toxic relatives of toxin genes have been identified (Hargreaves et al. 2014a). For example, genes encoding complement c3 and nerve growth factor have been shown to have undergone an Elapid-specific gene duplication (Sunagar et al. 2013; Hargreaves et al. 2014a, b) to give rise to the putatively toxic "cobra venom factor" and nerve growth factor b (Hargreaves et al. 2014b). This mis-identification of physiological orthologs as toxin-encoding paralogs has led to the conclusion that all Toxicoferan reptiles produce toxins in their oral secretions, and are therefore descended from a common venomous ancestor. In addition, many previous studies (e.g., Casewell et al. 2012) have been based on a flawed assumption – that phylogenetic trees containing monophyletic clades of *reptile sequences* that include a known (or hypothesised) toxin from venomous snakes constitute *venom toxin clades*. The true evolutionary history of these genes (which have duplicated to possibly give rise to toxic versions in *some* species), and these clades (which contain both genes encoding toxic products in *some* species, along with related genes encoding non-toxic products in other species), has therefore been obscured by being labelled as toxins by default. This is further confounded by a lack of data, both for the tissue being studied and also for other tissues and species (the majority of Toxicofera-related studies (Fry et al. 2006, 2010, 2012a) used only "up to 384" individual venom gland cDNA library colonies per species, a minimal amount of sequencing considering the frequently cited complexity of snake venoms (Li et al. 2005b; Kini and Doley 2010; Casewell et al. 2013)). This paucity of data, whilst understandable given the technology and resources of the time, has seemingly led to errors of interpretation, and, possibly more seriously, over-interpretation of results. Indeed, few genes were found expressed in all species surveyed (for example out of nine genes, only Kallikrein was detected expressed in the mandibular salivary gland of all four species of varanid (Fry et al. 2006)). With increased taxon sampling, only Kallikrein and CRISP were detected in all 18 species of lizard sampled (Fry et al. 2010) which included 13 species of varanid. Whilst this may be an artefact of low sequencing depth, the lack of consistent expression should have precluded these genes being used to support a conserved repertoire of "venom" genes across the Toxicofera.

Perhaps the most significant issue with the evidence used to support the Toxocifera hypothesis is that all samples used for sequencing were derived from either salivary or venom glands, and no "body" tissues were included with which to compare gene expression. Transcriptomic analysis of solely venom gland is perfectly

acceptable for descriptive studies which seek to characterise the transcriptome of this tissue. However, in order to assign a potential toxic role to a gene (and especially to infer its true evolutionary history, or the evolution of the venom repertoire in an entire lineage), sequencing the venom gland alone is insufficient. It has long been known that tissues all express a repertoire of "housekeeping" or maintenance genes (Butte et al. 2001) and as a result the sequencing of the entire venom or salivary gland will result in the identification of genes associated with a diverse range of functions (e.g., protein synthesis, cell-cell signalling and energy metabolism), not to mention that the sample will likely contain traces of other tissues such as muscle and blood. Consequently, genes cannot be inferred to encode toxins simply because they happen to be expressed in the venom or salivary gland.

Conservation in the structure of proteins detected in lizard oral secretions has also been used in support of the Toxicofera hypothesis. However, many secreted proteins, particularly members of the same gene family, have a conserved cysteine-rich "scaffold" (Anantharaman et al. 2003). It should not be too surprising that related proteins have similar structures, especially as alterations to this scaffold, or to the conserved residues, would likely result in a disruption of the protein structure and function. Similarity of structure should not necessarily always be considered to reflect shared toxicity. When using the Australian snake venom detection kit, Jelinek et al. (2004) found cross-reactivity between several snake species, most notably the tiger snake (*Notechis scutatus*) and the black-headed python (*Aspidites melanocephalus*). This has been used as evidence that putative toxin genes are translated into proteins in the venom or oral glands of these species, and that these proteins represent relics of an ancestral venom system which has been down-regulated in Henophidians (boas, pythons and several other families of basal snakes) (Fry et al. 2013). However, such cross-reactivity has been observed many years previously, with cross-reactivity demonstrated between colubrid oral secretions and antivenoms raised against African and Australian elapids (Minton and Weinstein 1987). Interestingly, the authors also found some antigenic cross-reactivity between a Henophidian snake (*Epicrates striatus strigilatus*) oral secretion when tested using a polyvalent antivenom raised against three *Dendroaspis* (mamba) species. Some of the responsible antigens were shown to be present in both venom and plasma, whilst some were present only in venom. Therefore, it is likely that some of this cross-reactivity between species is due to antigens present in secretions common to many species, as well as to cross-reaction between related members of protein families and cannot be taken as representative of any shared toxicity.

Whilst several Toxicofera-related studies commendably attempted to functionally test the oral secretions of some varanid lizard oral secretions, the results must be interpreted carefully. Purified group III PLA_2 from *V. varius* appears to have caused inhibition of platelet aggregation, although it is unclear why this was tested on human blood instead of the blood of native prey items such as birds or rabbits (Weavers 1989). It is also unclear whether physiological concentrations (within a range of concentrations which occur naturally in oral secretions) of this protein were used in this assay or if an increased dosage was required to achieve this inhibition of platelet aggregation.

Crude mandibular oral secretion and synthesised natriuretic peptide from *V. varius* and *V. komodoensis* caused a drop in mean arterial pressure when injected intravenously into anaesthetised rats (Fry et al. 2006, 2009). However, intravenous (I.V.) administration is an unlikely delivery method in the event of a lizard bite, and the depressor effects of I.V. administration of saliva has been noted in previous experiments (Gibbs 1935; Levy and Appleton 1942). Therefore, physiological effects noted in a controlled laboratory experiment may not be translated in a real life scenario. For crude *V. varius* mandibular secretion, a concentration of 1 mg kg^{-1} was required to cause a drop in blood pressure in an anaesthetised rat (Fry et al. 2006) whilst a decrease in blood pressure was seen at doses above 100 μg/kg for synthesised natriuretic peptide (from *V. komodoensis*) with 400μg/kg required to induce hypotensive collapse (Fry et al. 2009). Conversely, in a similar experiment, 10μg/kg of crude Papuan taipan (*Oxyuranus scutellatus canni*) venom caused a complete respiratory and cardiovascular collapse (Crachi et al. 1999). It is safe to say that lizard "venom" is much more inefficient, and coupled with the inefficient delivery method in these species, is it realistic that they will administer sufficient amounts of toxin in a single bite?

Casting Doubt on the Toxicofera Hypothesis

The Toxicofera hypothesis has been widely accepted for almost a decade, and has proved to be pervasive and attractive. However, the downside of these qualities is that it has also avoided scrutiny and testing. There have recently been several studies which have cast doubt on the Toxicofera hypothesis (Hargreaves et al. 2014a; Reyes-Velasco et al. 2015), although their interpretation has led to alternative conclusions. Several phylogenetic analyses incorporating non-venom gland transcriptomic data have shown that non-toxin sequences nest within clades of toxin genes, and it has been acknowledged that such findings provide "…strong evidence for the non-monophyly of Toxicoferan toxins" and that "…the results of [these] phylogenetic analyses would strongly refute the key prediction of the 'SEO' (single early origin) hypothesis…" (Casewell et al. 2012). Rather than accepting these conclusions, it has instead been proposed that venom gene recruitment may not be one-way, and that genes encoding venom toxins undergo a dynamic to-ing and fro-ing between toxin and physiological protein, whereby a venom toxin may undergo additional duplication, with subsequent recruitment back into a body tissue to fulfil a non-toxic physiological role. However, the more parsimonious hypothesis that these sequences actually represent *reptile* body sequences (which have never been toxins) forming *reptile* clades rather than body sequences nesting within *venom* clades is not considered. Similarly, Koludarov et al. (2012) investigated the oral secretions of the lizard *Abronia graminea* and determined that "the NGF [nerve growth factor] expressed in venom may be the same gene as is used in the body and therefore may be a rare case of a venom protein resulting from a non-duplicated gene." It is possible that the product of a gene may be used pleiotropically as a toxin (fulfilling a toxic and non-toxic role simultaneously), but unless its expression is

elevated in the salivary gland, there would be little evidence to suggest that it was anything more than a non-toxic physiological protein encoded by a housekeeping or maintenance gene.

More recent analyses incorporating an increased number of non-venom gland samples has further cast doubt on the Toxicofera hypothesis. A large scale test of the robustness of this hypothesis found that many of the genes used to support the single, early evolution of venom in squamates are in fact expressed in multiple body tissues including the salivary gland of a non-Toxicoferan lizard, the leopard gecko (*Eublepharis macularius*) (Hargreaves et al. 2014a). No evidence has been found of either a venom-specific splice variant or significantly elevated expression level in the venom or salivary gland. Therefore, it is likely that these genes are simply encoding maintenance or "housekeeping" proteins, and are expressed in multiple tissues at low levels. Many of these genes were also found expressed in several other body tissues in *Echis coloratus* (Hargreaves et al. 2014b), adding further support that these are housekeeping genes due to their ubiquitous expression pattern. Several of these genes are also only present as a single copy in the genome of this species, and so there is no evidence of duplication and recruitment of a toxic version to the venom gland (Hargreaves et al. 2014a). Indeed, genes homologous to known toxins have been found expressed in the rictal gland, brain, intestine, kidney, testes, spleen, ovary, heart, stomach, liver, blood and muscle of the Burmese python (*Python molurus bivittatus*) and the venom gland, liver, pancreas, kidney, brain and heart of *Bothrops jararaca* (Junqueira-de-Azevedo et al. 2014; Reyes-Velasco et al. 2015). Whilst these results have been interpreted in different ways, they demonstrate that genes which are homologous to putative venom genes are expressed in many different tissues outside of the oral glands, and that sequencing solely the venom or salivary gland without other body tissues to use as a reference for gene expression is not enough. Interestingly, when the genome of the Burmese python was surveyed for genes orthologous to putative toxin genes, only one or two orthologs were detected for each toxin gene family. The authors suggest that the Burmese python is representative of the ancestral state, prior to the expansion of toxin gene families in the Caenophidia (Reyes-Velasco et al. 2015).

If the proteins encoded by these genes are not being used to fulfil a venomous function, why are they still being expressed in the oral secretions of these reptiles? Given the metabolic cost of producing venom (McCue 2006) it would be more logical that natural selection would act to end any unnecessary gene expression and protein synthesis. Indeed, this process has been shown to occur in the marbled sea snake, *Aipysurus eydouxii*, following a switch in diet from fish to sedentary fish eggs (Li et al. 2005a, b), whereby several toxin genes have become pseudogenized (rendered non-functional via mutation). Why then has this not occurred in a plethora of reptile species which have no use for venomous function? Since many of the proposed toxins secreted by these glands are nothing of the sort, these oral secretions and the proteins they contain must have alternative functions, incorporating aspects of lubrication, pre-digestion and the stimulation of digestive processes and antimicrobial activity (Weinstein et al. 2012).

Glands and Fangs

Reptiles possess many salivary glands that secrete into the oral cavity, with a key role in the lubrication of food. Many are mucous in nature, however, some glands also have serous secretions which, in some cases, have become adapted as venom producing glands, as observed in venomous (Helodermatid) lizards, front-fanged snakes and some rear-fanged snakes.

In front-fanged snakes (such as elapids and vipers) and rear–fanged snakes, the fang and venom gland develop from a region at the back of the maxillary dental lamina (Vonk et al. 2008). The final position of the fangs is therefore attained by movement of the growing fangs, forward or backwards in the mouth, after initiation. Importantly in the venomous snakes, the venom gland and the fang appear to form from a united primordium that starts as an epithelial thickening below the eye on the upper jaw. This thickening has been called the primitive dental ridge (Martin 1899). In *Vipera palaestinae*, the thickening splits into an anterior gland and more posterior fang, with the venom gland extending first anteriorly before turning posteriorly and branching (Kochva 1963). In contrast to the serous venom gland, the nearby supralabial glands develop from independent placodes and are generally mucous.

In the rear-fanged snakes (Colubridae) the fang is associated with the Duvernoy's gland, which appears not to act as a venom gland and has instead been proposed to have an anti-bacterial role in coating dental surfaces (Jansen 1983). Secretion from the Duvernoy's gland in *Thamnophis elegans vagrans* was found to have enhanced anti-bacterial properties when compared to supralabial glands (Jansen 1983). In addition to a similar position of the fang primordium when compared to front-fanged snakes, the fang and venom gland of rear-fanged snakes also develops from a united primordium, as has been described in the opisthoglyph *Telescopus fallax* and aglyph *Thamnophis sirtalis* (Kochva 1965). *Telescopus* has a complete row of maxillary teeth with the fang primordia and gland forming at the posterior end. In contrast to the viperidae the venom gland does not first grow anteriorly before growing posteriorly. The fact that in these different snakes the venom gland and fang initiate from a common primordium that forms at the back of the maxillary dental lamina indicates that these front and rear fangs are homologous structures (see also Vonk et al. 2008). Importantly, Duvernoy's glands do not appear to form at all in many colubrids, for example some species of the genus *Elaphe*, genera *Lampropeltis*, *Pituophis*, *Pseuetes*, *Rhinocheilus* and *Spilotes* (Taub 1967). A variety of *Elaphe* species used in this study (although some of these have since been assigned to different genera) have no Duvernoy's gland and their supralabial glands are purely mucous (Taub 1967). In general such snakes without a Duvernoy's gland are constrictors who suffocate their prey before digestion. The lack of large serous glands in these species has been suggested to be due to secondary loss (Underwood and Kochva 1993; Vidal 2002). Although this may well be correct in some derived forms it is also possible that the Duvernoy's gland may not have evolved in all snakes, indicating independent evolution of this gland. Supporting this idea, Boidae and other primitive snakes have mainly mucous salivary glands, which are found at a range of positions in the oral cavity (Kochva and Gans 1970) In Boidae, anterior

temporal glands composed of serous cells have been described at the back of the maxilla (Taub 1966). Supralabial glands are generally thought of as mucous in most snakes but some Colubrids have serous cells included in the supralabial glands (Taub 1967). Thus whether a gland is mucous or serous is subject to some variation across reptiles and, in keeping with this, Duvenoy's glands can be mucous in part in some Colubridae (Taub 1967). Whether a gland is serous or mucous, therefore, cannot be necessarily used to infer evolutionary relationships.

In both front and rear fanged snakes, the fangs are associated with a gland that forms from the same dental primordium as the tooth. These are true dental glands. Any homologous structures would consequently be proposed to share this joint origin. It is therefore important to know whether venom glands in Toxicoferan lizards also develop from a united dental placode. If not, they are unlikely to be homologous, but instead would represent independent adaptations to venom formation in other oral glands. Some oral glands in lizards do indeed appear to develop from a lamina linked to the dental lamina. For example in chameleons the tooth and dental gland appear to share a similar origin (Tucker 2010). However in helodermatids, where venom glands are found on the lower jaw, the glands lie adjacent to the tooth with the duct at a slight distance (Kardong et al. 2009), indicating that the tooth and gland develop from separate placodes. Supporting this view, the ducts have been proposed to run to an opening between the lip and the jaw, rather than to the base of the teeth (Shufeldt 1891) and the location of the gland appears more similar to an infralabial gland. If this is the case, then the venom glands of helodermatids are not homologous to those of snakes.

The lack of a developmental link between dental glands and teeth in venomous lizards compared to snakes, and the lack of a large serous gland associated with the maxillary dental lamina in primitive snakes and some colubrids strongly suggests that the venom delivery system in snakes and lizards evolved independently. From the presence of Duvernoy's glands in snakes without venom, it would appear that the Duvernoy's gland first evolved as a branch of the forming dental lamina and then was adapted into a venom-producing gland in both front and rear-fanged snakes. A clear understanding of the embryonic development of the venom glands in venomous lizards will be important to clarify such points.

Varanid Venom

Many Toxicofera-related studies suggest that lizards belonging to the genus *Varanus* are in fact venomous, in particular the Komodo dragon *V. komodoensis* (Fry et al. 2006, 2009, 2013). A review of the available evidence found it unlikely that the Komodo dragon utilises venom as a prey capture method, instead suggesting that if it did use venom it was used as a pre-digestion method (Arbuckle 2009). Historical field observations have suggested that blood loss due to injury is the main prey capture strategy utilised by Komodo dragons (Auffenberg 1981). Whilst many *Varanus* species have been kept in captivity for many years, there have been almost no reports of any symptoms concurrent with envenomation following a bite. In the

original Toxicofera paper (Fry et al. 2006) there are anecdotal reports of bites from three species of *Varanus* which resulted in symptoms such as dizziness and rapid swelling. Most recently, a bite by a Bengal monitor (*Varanus bengalensis*) reportedly caused acute kidney injury to a human patient, which ultimately (and most unfortunately) resulted in death (Vikrant and Verma 2014). However, no positive identification was made of the offending animal, other than the name given by the patient. Perhaps more dubious is that the bite symptoms were more in line with envenoming from a Russell's viper (*Daboia russeli*) (White and Weinstein 2015), a member of the so-called "Big four" and a main cause of mortality due to snakebite in India (Simpson and Norris 2007). Unfortunately no mention is made of the bite wound itself which may aid in distinguishing between a lizard or snake as the culprit. Additionally, a recent bite by a Komodo dragon reportedly resulted in no symptoms of envenomation (Borek and Charlton 2015). Therefore, the status of varanid lizards as venomous is uncertain, particularly when compared to known venomous lizards such as the Gila monster and beaded lizards.

Conclusions and Future Directions

Venom Evolved Multiple Times in Reptile Evolution

Whilst the Toxicofera hypothesis represents a parsimonious explanation of the evolution of venom in reptiles (one character evolving a single time), the inclusion of non-venom-gland derived transcriptomic data in phylogenetic analyses along with the quantification of gene expression would strongly suggest that the Toxicofera hypothesis is unsupported (Hargreaves et al. 2014a). This would prompt a move back to the previous hypothesis that venom has evolved multiple times within squamate reptiles, once in the advanced snakes, once in the helodermatid lizards, and potentially another time in varanid lizards (although more evidence is needed to confirm this). This is in keeping with the large phylogenetic distance between venomous snakes and venomous lizards, the differing morphology of venom delivery systems between these animals (e.g., gland location, teeth/fangs), and the differing uses for their venoms (i.e., snakes predominantly for prey capture and helodermatid lizards for defence).

Simplified Complexity of Reptile Venom

The rejection of the Toxicofera hypothesis and the ruling out of many of the genes used to support it as toxins leads to an inescapable conclusion, that snake venom is not as complex as previously suggested (Li et al. 2005b; Kini and Doley 2010; Casewell et al. 2013). A review of venom proteome data from several species (Calvete et al. 2007; Wagstaff et al. 2009; Vonk et al. 2013) shows that snake venom is composed of a relatively small number of gene families encoding a few dozen different proteins, with most extensive diversity found in only one or a few of

these families (Calvete 2013; Hargreaves et al. 2014a). Whilst post-translational modifications may prove to play a significant role in generating more extensive diversity from a limited genetic background (Casewell et al. 2014), the idea that snake venom is a "complex cocktail" (Casewell et al. 2013) of hundreds of different proteins encoded by many gene families seems to be unsupported by experimental evidence. The low number of products in snake venom makes perfect sense as (1) a complex proteinaceous mixture would be metabolically expensive to produce and (2) natural selection will act to streamline the venom, tailoring it to the snakes' prey items. In short, a simple venom is efficient; a complex venom is overkill. The implications of this reduced complexity are significant, particularly for the development of the next generation of antivenom treatments utilising methods such as "string of beads" (Whitton et al. 1993) and "epitope-string" (Casewell et al. 2013). A reduction in the number of likely toxins inherently means a reduction in the number of targets requiring neutralisation by antivenom, and as a consequence the reduced number of components contained in the antivenom would mean a reduction in antigenicity, meaning a reduced chance of adverse reactions to treatment such as anaphylaxis and serum sickness (Nuchprayoon and Garner 1999).

From an evolutionary perspective, the reduction in the number of toxins does not detract from the fascination or specialization of venoms, in fact the opposite is true. The occurrence of lineage-specific gene duplications (for example *complement c3* and *nerve growth factor* in Elapids (Sunagar et al. 2013; Hargreaves et al. 2014a; Hargreaves et al. 2014b)) would indicate that these genes may confer some prey-specific effects (as seen in the Mangrove catsnake, *Boiga dendrophilia* (Pawlak et al. 2006)), or may have allowed adaptation to a new ecological niche.

The Changing Definition of Venom

The Oxford English dictionary defines venom as "a poisonous substance secreted by animals such as snakes, spiders, and scorpions and typically injected into prey or aggressors by biting and stinging." A more specific and long-standing definition would be "a complex substance produced in a specialized gland and delivered by an associated specialized apparatus that is deleterious to other organisms in a given dosage and is actively used in the subjugation and/or digestion of prey and/or in defence" (Mebs 2002). More recently, the quest for a catch-all term that encompasses the diverse uses of venom by insects, molluscs, reptiles and mammals has led to increasingly broad definitions of venom, such as "a secretion, produced in a specialised tissue (generally encapsulated in a gland) in one animal and delivered to a target animal through the infliction of a wound (regardless of how tiny it is). A venom must further contain molecules that disrupt normal physiological or biochemical processes so as to facilitate feeding or defence by/of the producing animal" (Fry et al. 2012b). It is perhaps time to discard this quest in favour of more restricted, possibly even lineage-specific, terminology with emphasis on the biological role of the venom to the survival of the animal. As an example, human saliva contains many of the proteins encoded by the same gene families which are also found present in the

snake venom proteome, including cystatins, disintegrin-like metalloproteinases, epididymal secretory protein E1, group IIA PLA_2s, β-defensins, and kallikrein (Hu et al. 2005; Guo et al. 2006). Human saliva has also been shown to be toxic (Bonilla et al. 1971). However, humans are not considered to be venomous, we do not use these secretions to kill or otherwise incapacitate prey, and so these proteins must fulfil some other biological role, such as pre-digestion and lubrication. Therefore, the presence of proteins homologous to known (or proposed) toxin proteins in oral secretions does not automatically mean that the organism is venomous. Moreover, considering the presence of homologous proteins in the oral secretions of basal snakes as toxins based on their use as toxins in more derived species, without evidence of these proteins showing any functional significance, is an erroneous and premature assumption, which has been stated previously by other authors (Kardong 2012; Weinstein et al. 2012).

Future Directions

The increased application of second generation DNA sequencing technologies and the integration of multiple types of 'omic data (genomic, transcriptomic, proteomic) is revolutionising the study of the evolution and composition of venom in reptiles, with implications not only for our understanding of this evolutionary innovation, but also for the treatment of snakebite and development of novel pharmaceuticals. Once the genome to proteome path of toxin expression is completely elucidated, this leaves the fundamentally important question: what do these proteins actually do? Perhaps more pertinent, is the functional property of these proteins relevant to the biological role of the venom and to the survival of the animal? Oral secretions are likely to have several biological roles, such as pre-digestion and lubrication, and so some proteins are likely to fulfil these rather than act as venom toxins. Only with functional characterisation (which can be a long and arduous task, particularly compared to the "one-shot" nature of high throughput sequencing) of these putative toxins can a true role be assigned to them. Moreover, functional testing of proteins should be performed at physiological concentrations on native prey items.

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Functional and Genetic Diversity of Toxins in Sea Anemones

5

Marymegan Daly

Abstract

Sea anemones are benthic, sessile cnidarians that use venom for prey capture, defense, digestion, and intraspecific competition. Lacking venom glands, sea anemones produce venom locally in the tissue of use and deliver it via subcellular structures called nematocysts. The majority of venoms characterized from anemones are unique to the lineage. Although there are many components of venom that are only known from particular lineages, these are generally not associated with structures that are unique to those lineages. The few kinds of venoms that have been explored in an evolutionary context appear to evolve under negative selection, although positive selection may occur on select residues within the molecule. Because there is a positive relationship between study effort and number of toxins known from any lineage, it is likely that broader taxonomic representation in studies of anemone venom will increase the number of genes and molecules reported from anemones.

Keywords

Cnidaria • Actiniaria • Cytolysins • Voltage-Gated Channel Toxins

Contents

M. Daly (✉)
Museum of Biological Diversity, The Ohio State University, Columbus, OH, USA
e-mail: marymegandaly@gmail.com; daly.66@osu.edu

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_17

Introduction

Cnidaria is the earliest-diverging metazoan lineage in which venoms have evolved and is the only phylum-level lineage for which venom and venom-delivery structures are synapomorphic (Daly et al. 2007). In addition to being the oldest venomous lineage, Cnidaria is the lineage showing greatest diversity in the known uses of venom, which is critical to prey capture, digestion, and intraspecific aggression (Doumenc and Van Praët 1987). Among cnidarians, venom is best characterized in sea anemones (Cnidaria: Anthozoa: Actiniaria). Sea anemone venoms have generally been studied one species at a time, with an emphasis on the toxicology and structure of the venom proteins. Recovering the complete venom and understanding the genotype-to-phenotype relationship in the multigene venom phenotype is especially complicated for sea anemones and other Cnidaria because there is not a single kind of venom or a single locus of venom production (e.g., a venom gland); venom is produced by the nematocysts and adjacent secretory cells within tissues (Basulto et al. 2006; Moran et al. 2011).

The evolution of venom in sea anemones can be understood through consideration of the evolutionary history of the lineages in which it occurs, of the ways in which it is used, or of the molecules themselves. These avenues are not independent but nonetheless provide different insights into the diversity and evolution of the complex venom phenotype in sea anemones.

Diversity and Phylogeny of Actiniarian Sea Anemones

Cnidarians are epithelial animals that produce cnidae, tiny intracellular capsules made of protein and collagen (Daly et al. 2007). The tubule, lying introverted within the capsule, everts upon stimulation; this action leads to a chemical or mechanical interaction in which the tubule punctures or entangles predators, prey, or debris. Cnidae vary across the phylum in terms of the anatomy of the capsule and tubule and have been classified as nematocysts (double-walled capsule, tubule penetrating or entangling, often containing venom), spirocysts (single-walled capsule, tubule entangling), and ptychocysts (single-walled capsule, tubule entangling) (Mariscal 1974). Nematocysts are produced by all cnidarians in cells called nematocytes. Spirocysts and ptychocysts are produced by spirocytes and ptychocytes, which are unique to subsets of Anthozoa. Although all cnidae are inferred to have a single origin, the relationship among the various kinds of capsules is unclear (Reft and Daly 2012). Nematocysts are assumed to be critical

to the delivery of venom in cnidarians and clearly contain some venom within the capsule (Balasubramanian et al. 2012; Moran et al. 2013), but nematocytes are not the only kind of cell in which toxins are made (Basulto et al. 2006; Moran et al. 2011).

Cnidaria comprises two reciprocally monophyletic groups: Anthozoa and Medusozoa (Zapata et al. 2015). Medusozoa contains four monophyletic lineages which have each been ranked as a class (Cubozoa, Hydrozoa, Scyphozoa, and Staurozoa). Anthozoa contains two daughter clades, Octocorallia and Hexacorallia; these are generally recognized as monophyletic, although the relative position of the hexacorallian order Ceriantharia has been problematic and some analyses place it as a sister to Hexacorallia and Octocorallia or even outside of the two primary groups within Anthozoa (reviewed in Rodríguez et al. 2014; Zapata et al. 2015). Although Anthozoa has historically been accorded status as a class, the diversity within it and the hierarchical structure of the group relative to that of Medusozoa have been offered as arguments for recognizing Hexacorallia and Octocorallia as classes rather than subclasses (Daly et al. 2007; Zapata et al. 2015).

Octocorals and hexacorals share the anthozoan attributes of being exclusively polypoid, having the mouth project into the gastrovascular cavity as an actinopharynx, and having sheets of endodermal tissue (mesenteries) that project from the body wall into the gastrovascular cavity. Octocorals generally have eight-part symmetry, with eight pinnate tentacles and eight internal mesenteries. Despite their name, hexacorals are varied in their symmetry, although 12 is the common denominator for the organization of tentacles and mesenteries in the majority of its lineages. Hexacorals are more diverse in polyp anatomy than any other lineage in Cnidaria and so have been hard to circumscribe (Daly et al. 2007), but they can be diagnosed by the shared production of spirocysts, the single-walled, entangling cnida. Diversity within hexacorals has historically been organized based on differences in polyp anatomy and skeletal production (Daly et al. 2007; Rodríguez et al. 2014). Analysis of DNA sequences has confirmed that the majority of the ordinal distinctions are robust (Rodríguez et al. 2014), although the discovery of new lineages remains a possibility in Hexacorallia (as for many other marine groups: Appeltans et al. 2012).

Sea anemones belong to the hexacorallian order Actiniaria. This group contains only solitary, sessile, benthic polyps. They are distinguished from other solitary and soft-bodied hexacorals in lacking the signature attributes of those other orders: they lack ptychocyst cnidae and have tentacles and mesenteries arranged unlike those of members of Ceriantharia, lack the distinctive arrangement of mesenteries seen in members of Zoanthidea, and have musculature and nematocysts that differentiate them from members of Corallimorpharia. Despite the challenge of defining Actiniaria relative to the other orders based on anatomical features, DNA sequence data are unambiguous in finding Actiniaria to be monophyletic with respect to these groups (see Rodríguez et al. 2014).

Sea anemones are exclusively marine, inhabiting oceans at all depths and latitudes. They are among the most conspicuous inhabitants of marine ecosystems,

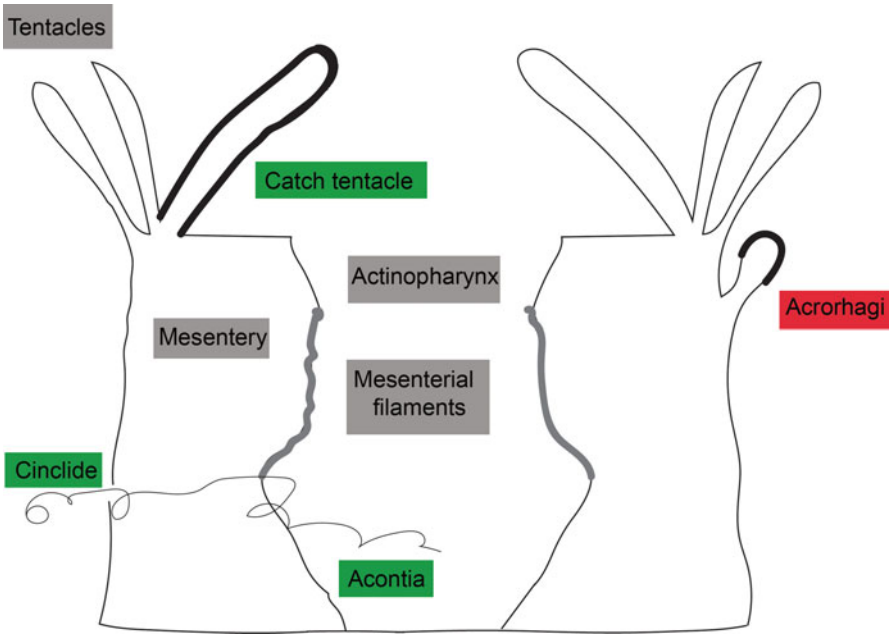

Fig. 1 Diagrammatic representation of sea anemone anatomy, highlighting structures relevant to the use of venom. The polyp is shown in longitudinal section. Tissues in *grey* are common to all anemones. Those in *red* (acrorhagi) are seen only in members of Actinioidea; those in *green* (acontia, catch tentacles, cinclides) are seen only in members of Metridioidea

where they play critical roles in benthic-pelagic coupling and, through their symbiosis with photosynthetic unicellular organisms, primary production (Shick 1991). Like all cnidarians, sea anemones have bodies that are two cell layers thick, with no organs and no centralized nerve, sensory, circulatory, or osmoregulatory apparatus. However, among cnidarian polyps, sea anemone bodies are complex in having specialized structures associated with digestion, reproduction, defense, and competition (Fig. 1). Many of these structures are defined by a specialized complement of nematocysts (Doumenc and Van Praët 1987). All of the nematocysts in sea anemones are "stommocnidae" (Mariscal 1974), with a hollow tubule that is inferred to penetrate the skin of the victim. The structure of the tubule within the nematocyst capsule, and thus the way in which the nematocyst is categorized, varies across tissue, ecology, and lineage. There is considerable species-level diversity in the structure of the tubule and thus in the "type" of nematocyst within any tissue, but at a broad-scale, the cnidom of each lineage within Actiniaria is similar, including spirocysts, basitrichs, holotrichs (= atrichs), microbasic *b*-mastigophores, and microbasic *p*-mastigophores (Rodríguez et al. 2014).

Relationships within Actiniaria as detected by DNA or modern analyses of morphological characters do not accord with the divisions of the traditional classification. The order has been revised (Rodríguez et al. 2014) so that taxonomic

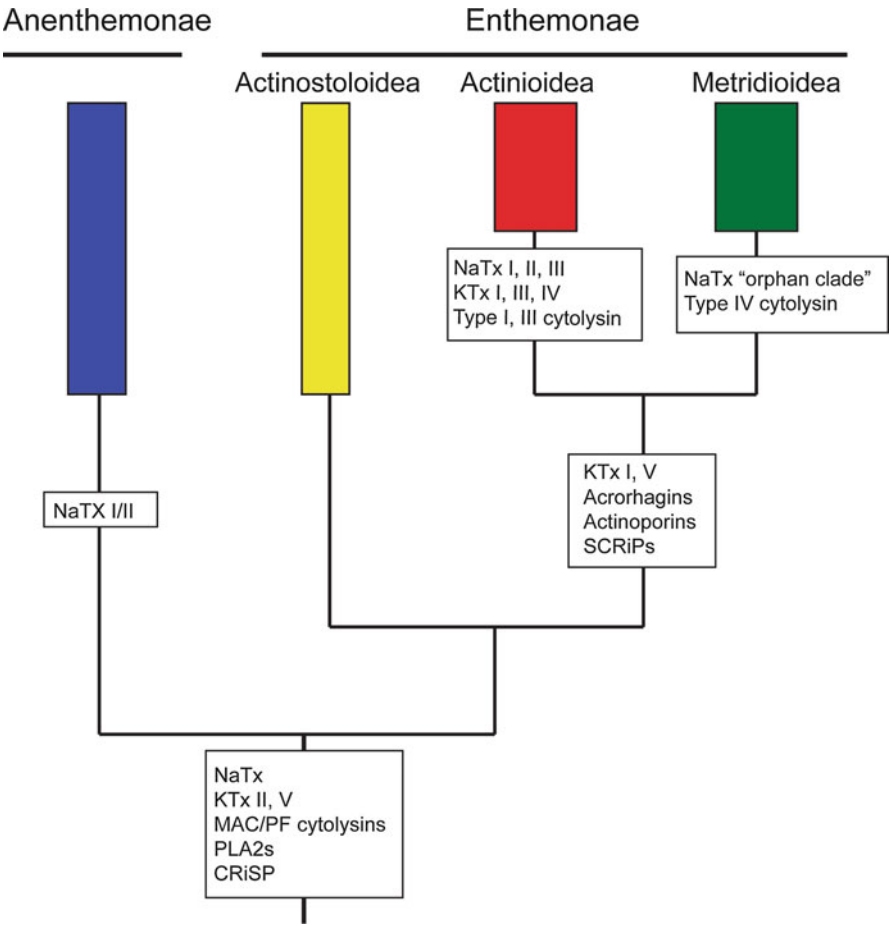

Fig. 2 Phylogenetic relationships among major lineages of sea anemones (After Rodríguez et al. 2014) with the reported occurrence of venoms. Venoms have been associated to the most general group possible and may not occur in all members of a lineage; for example, type I cytolysin is known from only a subset of Actinioidea. Preliminary evidence suggests that Type II and IV cytolysins may be common to both Actinioidea and Metridiodea (see text); these have not been fully characterized or analyzed. Given the current state of knowledge, the localization of each of these is subject to change; in particular, those venoms that are associated with the common ancestral lineage for Actinioidea + Metridioidea may be shared by all Enthemonae. The occurrence of some kind of NaTx in all anemones suggests that the venome of the common ancestor to all sea anemones included NaTx, but this ancestral molecule likely does not fit into the existing scheme of classification for NaTx

divisions correspond to phylogenetic relationships (Fig. 2). Thus, the primary division within the order is between the Anenthemonae and Enthemonae. Anenthemonae is the less species-rich of the suborders, containing members of families Actinernidae, Edwardsiidae, and Halcuriidae; the model organism

Nematostella vectensis is the most familiar and well-studied member of this group. Enthemonae contains the overwhelming majority of species and anatomical diversity within Actiniaria and is further subdivided into three superfamilies, Actinioidea, Actinostoloidea, and Metridiodea (Fig. 2). These correspond roughly to the traditional divisions between Endomyaria, Mesomyaria, and Acontiaria, respectively, although the exact composition of Actinostoloidea and Metridioidea differ somewhat from the circumscriptions of Mesomyaria and Acontiaria.

Diversity and Evolution of Structures and Behaviors Associated with Venom in Sea Anemones

Venom is fundamental to the biology of sea anemones. It is part of feeding, defense, and intraspecific competition, a broader repertoire than reported for any other venomous lineage (see e.g., Casewell et al. 2013). Because sea anemones do not have a central venom gland or venom delivery surface, there is the potential for functional specialization of venom in particular tissues based on the function of that tissue. The functional specialization of the sea anemone body is associated with differences in the size and distribution of nematocysts and many regions of the body are limited in their function (Doumenc and Van Praët 1987). Although no tissue-specific venom phenotype (as a protein profile) has been published for any species of anemone, assessments of venom based on mucus (e.g., Rodríguez et al. 2012), gene expression (e.g., Orts et al. 2013), and transcriptomes (e.g., Macrander et al. 2015a) suggest that there are species- and tissue-specific differences in the venom phenotype. However, function does not map one-to-one with particular tissues or body parts, and the absence of information about a toxin within a tissue should be interpreted cautiously in light of the incompleteness of knowledge about the occurrence of venom in sea anemones.

Although they are flexible in the ways in which they obtain nutrition (Shick 1991), sea anemones are fundamentally predatory animals, using their tentacles to catch prey. Because they lack true muscle tissue, have no visual capacity and lack a centralized or coordinated nervous system, prey capture relies heavily on toxins to subdue prey. Adhesion of prey to tentacles relies on spirocysts, cnidae which are interpreted as adhesive rather than penetrating and which have not been shown to contain venom (Doumenc 1971). Although spirocysts are the most abundant kind of cnida in the tentacles of sea anemones, penetrating, venom-bearing cnidae are also found in tentacles – generally basitrichs and often microbasic p-mastigophores. In addition to the effect of venoms injected by nematocysts, the same or different toxins produced by gland cells (Basulto et al. 2006; Moran et al. 2011) probably render the copious mucus of sea anemone tentacles effective as a paralytic agent.

Venom is also inferred to have a role in the digestion of prey. Like all cnidarians, sea anemones lack a stomach, using the gastrovascular cavity as a holding space for prey. Food is digested within the cavity and nutrients are absorbed across the gastrodermal epithelium. Lacking teeth, muscular gizzards,

or any other means of mechanical degradation of food, cnidarians rely on chemical digestion. Cytolytic toxins in the tentacles may predigest food. The numerous gland cells and nematocysts (primarily microbasic *p*-mastigophores, but also basitrichs and microbasic *b*-mastigophores) of the mesenterial filaments are inferred to be important in the lysis of food items. Cytolytic toxins that form pores in the membranes of non-anthozoan tissues have been identified in the mesenterial filaments (Meinardi et al. 1995; Basulto et al. 2006) and in the actinopharynx (Moran et al. 2011). Although ubiquitous in cell membranes of eukaryotes, sphingomyelin is not present in the membranes of sea anemones; this innovation likely protects sea anemones from self-digestion (Meinardi et al. 1995). Whether all of these are venoms injected by nematocysts or simply digestive toxins remains unclear.

Nematocysts and toxins in the mucus also play a role in defense. Tentacles are used directly in agonistic interactions with potential predators; the venom contained within or produced by nearby gland cells is inferred to be of primary importance in these interactions (Hines and Pawlik 2012). Neurotoxins have been identified in planula larva and juvenile polyp of *Nematostella vectensis* (Orts et al. 2013) and are interpreted to have a defensive function. Nematocysts and toxins are key to protecting photosynthetic microorganisms, fish, and crustaceans that associate with anemones (Shick 1991; Mebs 2009). The nematocyst complement of sea anemones that host photosynthetic organisms are not different from that of closely related species that are aposymbiotic, bolstering the contention that capacity for harboring photosymbionts is primitive in many lineages (Geller and Walton 2001) and perhaps a primary function of the tentacle cnidom. For mobile animal symbionts that live on the outside of the body rather than within the host tissue, the protection afforded by nematocysts is a double-edged sword. The symbionts may "hide" themselves from the triggering mechanisms of the nematocysts by behavioral and chemical means, including coating themselves in the host's mucus. However, instances of toxin resistance have been reported in fish that engage in species-specific symbioses with anemones, and the vulnerability of some fish changes over developmental time in other cases, suggesting that coevolutionary changes in venom and receptivity may occur (Mebs 2009).

All modern studies of the interaction between host anemones and their symbionts to date have focused on host species belonging to Actinioidea (e.g., members of Stichodactylidae, *Entacmaea* spp., *Condylactis* spp.). However, symbioses with crustaceans are well characterized in several lineages within Metridioidea and in a few species within Actinostoloidea (Ross 1974; Vader 1983). The means of circumventing envenomation and the nature of coevolutionary changes in those lineages are unknown. Laboratory studies of the effects of toxins from *Adamsia palliata* and *Calliactis parasitica*, sea anemones that live in association with hermit crabs, attest to its efficacy on crustacean targets (Mebs 2009). The toxicity of anemone venom to crabs underpins the behavior of *Lybia edmonsoni*, a xanthid crab that fight conspecifics using sea anemones (*Triactis producta*) held on their chelae (Karplus et al. 1998). Although the exoskeleton clearly protects crustaceans

from the host anemones, coevolution may also play some role in the resistance of symbiotic crustaceans to the venom of their host sea anemone. Early studies by Cosmovici (1925) and Cantacuzene (1925) found that crude extract from the anemone had limited or no effects on host species and indirectly implicated crab host hemolyph as the source of this resistance.

Defensive venom in metridiodeans is produced in the tentacles and column and also in acontia (Fig. 1), extensions of the mesenterial filaments that can be extruded through special pores called cinclides or ejected through the mouth. Acontia are comprised primarily of nematocysts; they also contain secretory cells, but these are less numerous than nematocysts and relatively less common in acontia than in mesenterial filaments. They are interpreted to act primarily as defensive structures but may also play a role in digestion (Shick 1991). Acontia are integral to the defensive response of the *Adamsia*, *Calliactis*, and the other hormathiid hermit crab anemones: when disturbed, the anemone emits these nematocyst-packed threads through the cinclides; predators are deterred by the sting that presumably accompanies contact with acontia (Ross 1971). Acontia emission is generally not triggered by interaction with the symbiotic crab. Although acontia are likely central to the protective effect of the anemone association with hermit crabs, they occur more broadly across Metridioidea and are inferred to be ancestrally present in most Metridioidea, arising after the split between *Triactis* (family Aliciidae), *Paranthus* (family Actinostolidae), and all other metridiodeans (Rodríguez et al. 2014). Acontia generally contain different types or sizes of nematocysts than the mesenterial filament, a difference that highlights the potential for functional differentiation between the filaments and acontia. In the evolutionary history of Metridioidea, acontia are inferred to evolve a relatively simpler cnidom through loss of microbasic *p*-mastigophores and to have been lost altogether, particularly in lineages whose members inhabit the deep sea. Innovations in the evolution of acontia include both loss and novel the incorporation types of cnidae into the acontia, loss of cinclides, and the loss of acontia (Rodríguez et al. 2014).

Sea anemones are among the small number of venomous animals known to use venom in agonistic interactions with conspecifics (Casewell et al. 2013). Intraspecific agonism in anemones relies on structures that differ depending on the lineage: actinioidean sea anemones use novel structures on the column called acrorhagi, whereas metridioidean sea anemones use modified tentacles, referred to as catch tentacles (Fig. 1). Both acrorhagi and catch tentacles are used only in agonistic interactions with conspecifics. Although these structures are not homologous to one another and each instance of intraspecific agonism is interpreted as a parallel innovation (Rodríguez et al. 2014), in both Actinioidea and Metridioidea intraspecific antagonism invokes holotrichous nematocysts (reviewed by Bigger 1988).

The anatomical distinctiveness of acrorhagi and their functional limitation to agonism have made them relatively easy targets for studies of tissue-specific venom. Early studies of these tissues lead to the discovery and characterization of acrorhagin, a kind of toxin so far known only from sea anemones (Honma et al. 2005). Investigation of the acrorhagi of *Anthopleura fuscoviridis* and *Anthopleura* aff. *xanthogrammica* led to the discovery of novel Kunitz-type protease

inhibitors (Minagawa et al. 2008). A transcriptomic assay of the acrorhagi of *Anthopleura elegantissima* recovered two kinds of acrorhagins, plus several genes that were candidate cytolysins (Type II & IV), phospholipase A_2s (PLA2s), voltage-gated potassium channel toxins (Types I, II, III, IV & V), and voltage-gated sodium channel toxins (Types I, II & III) (Macrander et al. 2015a).

The venom of catch tentacles has not been studied specifically, perhaps because these structures are difficult to differentiate from feeding tentacles. However, among the acrorhagi-expressed cytolysins identified by Macrander et al. (2015a) is a gene that is highly similar to a gene retrieved from *Sagartia rosea*, a metridioidean belonging to a genus whose members produce catch tentacles. Furthermore, Macrander et al. (2015a) found a gene copy highly similar to the presumed to be acrorhagi-specific acrorhagin in the transcriptome of *Metridium senile*. The function of the acrorhagin-like product in *Metridium* has not been identified and no phylogenetic analysis has shown that this is a homolog of acrorhagin (and if so, how it is related to the acrorhagin of *Actinia*, *Anthopleura*, etc.). These similarities in venom highlight the potential for deeply conserved toxins that could be invoked in parallel in agonistic interactions.

Diversity and Evolution of Toxins Within Sea Anemones

Research on venom constituents in sea anemones has focused on characterization of the structure and function of the toxins, with much less attention to the ecology and evolution of the molecules. Even in those areas where there has been significant research effort, the venom of sea anemones is imperfectly known. Conclusions about its composition, its biological role, and its evolution remain highly contingent. Many toxins currently known from only a few species or from only a single lineage may be more widely distributed than currently appreciated. Innovations in proteomics, transcriptomics, and genomics promise to increase the number of species for which toxins are characterized and thus will facilitate the comparative study of the genes and proteins that make venom (Sunagar et al. 2016).

At a broad scale, the venom of sea anemones is quite distinct from that of medusozoan cnidarians (Rachamim et al. 2015). Many of the key constituents of sea anemone venom have no known ortholog outside of anemones. The functional characterization of the venome of each major cnidarian lineage suggests that Anthozoa and Medusozoa have contrasting strategies for their use of venom, with anthozoans (or at least actiniarians) relying much more on neurotoxins than do medusozoans (Rachamim et al. 2015). A similar conclusion obtains in comparisons within Anthozoa: nematocyst-mediated defense (venom) is more critical to sea anemones than to hexacorals of orders Corallimorpharia and Zoanthidea (which rely on toxins that are not injected: Hines and Pawlik 2012).

Contrary to expectations of repeated recruitment of venom genes from broadly-shared and physiologically diverse genes (e.g., Fry et al. 2009), the majority of venom genes in sea anemones are unique to this lineage. This emphasis on taxonomically restricted genes in venom seems to be more common among ancient

lineages of venomous animals (Sunagar and Moran 2015). The few elements of the venom of sea anemones that have a counterpart in the venom of other animals (e.g., protease inhibitors, CRISPs) have ambiguous roles, making comparison problematic in the absence of detailed functional study. For those toxins unique to anemones, only the voltage-gated and cytolytic toxins have been explored in an evolutionary framework.

Voltage-Gated Toxins

Voltage-gated sodium channel toxins (NaTx) are among the most diverse toxins described in sea anemones. These polypeptides range in size from 3 to 5 kDa and inhibit the inactivation of voltage-gated sodium channels (reviewed in Moran et al. 2009). Homologs of NaTx have not been identified in other cnidarians. These toxins or genes that are expected to encode them have been found in assays of tentacles and acrorhagi and in whole-animal assays. They are produced in planula larvae of *Nematostella* and are also present in the oocytes of *Anemonia viridis* (Moran et al. 2009), suggesting that they play a role in defense. In the genome of *Nematostella*, at least eight of the genes encoding NaTx are physically near one another and potentially transcribed as a single unit (Moran et al. 2008). Although the multiple copies presumably represent at least some ancient gene duplications, because of concerted evolution, some gene copies within a species may be more similar to one another than to their orthologs in other species (Moran et al. 2008, 2009). Although this is likely generally true for NaTx in anemones, not all NaTX transcripts show concerted evolution (Macrander et al. 2015b).

All three types of NaTx are reported from Actinioidea; many fewer NaTx have been reported for metridiodeans (Jouiaei et al. 2015) (Fig. 2). Although proposed as different based on early studies of species of *Heteractis* and *Anemonia*, the distinction between Type I and Type II NaTx is less clear-cut when toxins from other lineages are considered (Ishida et al. 1997; Moran and Gurevitz 2006): the NaTx of the anenthemonaeans *Nematostella vectensis* and *Halcurias* sp. seem to be precursors to the Type I and Type II NaTx of enthemonaean anemones. This pattern suggests that some kind of NaTx was made by the common ancestor of sea anemones (Fig. 2).

Because they have no homolog outside of Actiniaria, interpretation of the evolution of NaTx in sea anemones is especially reliant on comparison of the gene and organismal phylogenies to root and contextualize the network of NaTx genes. Rooting a tree of NaTx sequences from *Nematostella* (Anenethemonae), *Calliactis* (Enthemonae-Metridiodea), *Actinia* (Enthemonae-Actiniodea), and *Anemonia* (Enthemonae-Actiniodea) with *Calliactis* rather than *Nematostella* (as done by e.g., Moran et al. 2009) is inappropriate given the relationships among these lineages (Fig. 2). A tree rooted on *Nematostella* (Macrander et al. 2015b) suggests that Type I and Type II NaTx underwent a duplication within Actinioidea and that the "orphan clade" of NaTx is the metridioidean sister to this large clade of NaTx gene sequences from actinioideans.

Voltage-gated toxins that attack the potassium channel (KTx) are more diverse in form and function than NaTX and are presumed to represent multiple independent origins from nontoxin molecules (Orts et al. 2013). Their orthologs outside of anemones are not known. All of these act to inhibit the activity of membrane potassium channels, with different types having affinity for different channels. As for NaTX, the five types of KTx are differentiated by their amino acid composition, folding pattern, and target site. Type I KTx are reported only from enthemonaean anemones (Frazão et al. 2012) (Fig. 2), where they may be diverse and abundant. Macrander et al. (2015a) find that the copies from *Anthopleura elegantissima* are not the result of a single radiation within this species. The gene phylogeny of type I KTx includes mostly sequences from actinioideans, but one sequence from the metridioidean *Metridium senile* is associated with these, suggesting widespread gene loss in that lineage or many as-yet undiscovered KTx in this lineage (Macrander et al. 2015a).

Types II, III, and IV KTx have only been reported from a subset of Enthemonae, the actinioidean sea anemones (Frazão et al. 2012). Type IV KTx have been reported only from *Stichodactyla haddoni,* although it bears some similarity to a toxin from *Heteractis aurora* (as *Antheopsis maculata*, Honma et al. 2005), and incomplete candidate sequences were also retrieved in the actinioidean *Anthopleura elegantissima* (Macrander et al. 2015a). The narrow known distribution of type IV KTx precludes phylogenetic analysis. Type II KTx acts both as voltage-gated potassium channel toxins and as Kunitz-type protease inhibitors (KPI; Minagawa et al. 2008). This dual function makes it difficult to make inferences about the likelihood of a gene being a functional toxin based on sequence data alone but may explain the repeated evolution of potassium channel toxins across animals (Fry et al. 2009). A gene phylogeny of type II KTx and KPI from sea anemones suggests that members of this gene family have undergone both ancient and recent duplications (Macrander et al. 2015a) and that there is a differentiation between those genes that act primarily as KPI and those that act as KTx, as the genes with known function fall into two clades. Jouiaei et al. (2015) find that Type I and Type II KTx both evolve under strong negative selection, although some sites on the surface of each molecule show evidence of positive selection. This evolutionary pattern may explain the diversity of copies within a species, as the combination of functional constraint and relaxed selection on surface sites may lead to neofunctionalization (Jouiaei et al. 2015).

Unlike the relatively narrowly distributed types I-IV KTx, Type V KTx occur in all major lineages of sea anemones (Orts et al. 2013). This is the only KTx reported in *Nematostella vectensis*, where its expression begins early in development in cells that appear to be nematocysts (Orts et al. 2013) (Fig. 2). It has also been reported in *Bunodosoma caissarum*, *Metridium senile*, and *Anthopleura elegantissima* (Orts et al. 2013; Macrander et al. 2015a). Because it occurs in all major lineages of sea anemone, Orts et al. (2013) hypothesize that this toxin has a very ancient origin, likely being part of the ancestral venome of sea anemones.

The focus on cellular target and function for the various voltage-gated toxins may obscure evolutionary similarity. In their analysis of the voltage-gated toxins

from sea anemones, Jouiaei et al. (2015) find that Type I NaTx and Type III KTx are most closely related to one another. Thus, from an evolutionary standpoint, type II KTx are highly modified NaTx. This phylogenetic result contextualizes functional and biochemical studies that had identified similarities between NaTX and type III KTx but stands in contrast to the inferred origin and history of functionally similar toxins in other animal lineages (e.g., Zhang et al. 2015). These toxins are also the only toxins in sea anemones that show strong evidence of evolving under positive selection (Jouiaei et al. 2015) and thus the only kind of toxin that conforms to the pattern of rapid, positive selection that has been proposed as the general mode of evolution of venom genes (reviewed by Casewell et al. 2013).

Cytolytic Toxins

Although all act to disrupt the cell membrane of target cells, cytolytic toxins in sea anemones are heterogeneous and diverse in terms of their structure and precise mode of action (Anderluh et al. 2011). In addition to forming pores in membranes, some cytolysins of sea anemones may act as antihistamines (Type I) or PLA2s (Type III). Most of the cytolysins identified to date are restricted in their phylogenetic occurrence and their evolutionary relationships to other cytolytic toxins in other lineages has not been investigated. Type I cytolysins have been reported only from *Heteractis crispa* (as *Radianthus macrodactyla*) (Monastyrnaya et al. 2002). Type III cytolysins are similarly restricted in their phylogenetic distribution, being reported only from members of the actinioidean genus *Urticina* (Anderluh et al. 2011). The type III cytolysins UPI and UCI were isolated from *Urticina piscivora* and *U. crassicornis*, respectively, and have cardiostimulatory properties (reviewed in Anderluh et al. 2011). *Exaiptasia pallida* has a cytolysin similar in size to UPI and UCI in the acontia (Maček 1992), but it is unclear whether this has functional or evolutionary similarity to the other type III cytolysins. Type IV cytolysins are the largest cytolysins and are characterized with respect to function and structure only in *Metridium senile*. Sequences similar to these were recovered in the transcriptome of acrorhagi from *Anthopleura elegantissima* (Macrander et al. 2015), indicating a broader taxonomic distribution and potentially greater functional capability than previously recognized.

Cytolysins that resemble membrane attack complex/perforins (MAC/PF) have been characterized in *Actineria villosa* and *Phyllodiscus semoni* (reviewed in Anderluh et al. 2011). These species are not thought to be closely related: *Actineria* is a member of Thalassianthidae, a family historically placed within Endomyaria and thus expected to belong to Actinioidea, and *Phyllodiscus* belongs to Aliciidae, a family whose members form the stem or sister group to Metridioidea (Rodríguez et al. 2014). However, both of these species are capable of delivering powerful stings, raising the possibility that the venoms are convergent or, given the superficial similarity of the species, that they have been misidentified.

MAC/PF proteins have not been identified as part of the venom in other sea anemones, but they occur in other species: at least four MAC/PF genes are present in the genome of *Nematostella* (Miller et al. 2007). A broad-scale analysis of the evolution of the complement system highlights that the MAC/PF genes in anemones are different from those of triploblasts in several respects (Kimura et al. 2009; Rachamim et al. 2015). The MAC/PF genes of sea anemones do not cluster with those of other Cnidaria and lie outside of a cluster that includes those genes that play a role in the animal immune system (Rachamim et al. 2015). The tree of MAC/PF genes from Cnidaria is very sparse in terms of taxon sampling, but if correct, it suggests a very ancient duplication of MAC/PF genes, with one branch contributing to the evolution of the immune system and the other becoming critical to the venome of sea anemones.

Type II cytolysins, or actinoporins, are the most common and best characterized type of cytolysin (Anderluh et al. 2011). These cytolysins recognize and bind to sphingomyelin (Bakrač and Anderluh 2010). These are only characterized for actinioideans and metridiodeans (Fig. 2). Although Meinardi et al. (1995) did not identify the toxin they found in the gastrovascular cavity of *Phymactis clematis*, the fact that it recognized sphingomyelin suggests that it is an actinoporin and thus that these play some role in digestion. Basulto et al. (2006) found antibodies to actinoporins in the tentacles and filaments of *Sticohodactyla helianthus*, a finding also consistent with a role in digestion. However, actinoporins were abundant in the transcriptome of acrorhagi of *Anthopleura elegantissima* (Macrander et al. 2015a) and this tissue is not used in prey capture or digestion.

Actinoporins have only been characterized from sea anemones and have no known ortholog outside of Actiniaria. An actinoporin-like molecule has been identified in the hydrozoan *Hydra*, but the relationship of this molecule to actinoporins from sea anemones is unclear (Rachamim et al. 2015). Because phylogenetic analysis of actinoporins has included only those actinoporins for which sequence (rather than protein or biochemical) data are available, the full diversity of toxins has not been considered. In their analysis, Frazão et al. (2012) find that the actinoporins from actinioidean anemones (members of *Actinia*, *Anthopleura*, *Heteractis*, *Oulactis*, *Stichodactyla,* and *Urticina*) form a clade and that a clade of sequences from metridiodeans (*Sagartia* and *Phyllodiscus*) plus *Actineria* is sister to this actinioidean cluster. Lineage-specific duplications have occurred in actinoporins within Actinioidea (Frazão et al. 2012; Macrander et al. 2015a). Frazão et al. (2012) also find that a second set of sequences from *Actineria* and *Phyllodiscus* lies outside of the clade that includes the metridioidean and actinioidean actinoporins. These cytolysins of *Actineria* and *Phyllodiscus* have a MAC/PF domain (based on sequence similarity) and are only distantly related to the other actinoporins; their phylogenetic relatedness to actinoporins should not be overinterpreted. In their study of the mode of selection acting on diverse cnidarian toxins, Jouiaei et al. (2015) found a general pattern of negative selection for actinoporins. Although some of their analyses support the inference of episodic diversifying selection (Jouiaei et al. 2015), examples of positive selection that would be required by this scenario are limited.

Other Venom Constituents

Phospholipase A_2s (PLA2s) are enzymes that contribute to the venom of diverse animals (Casewell et al. 2013). They are found in the venom of members of all lineages of sea anemone (Nevalainen et al. 2004; Frazão et al. 2012). The PLA2s characterized from sea anemones are diverse and thus difficult to classify in a meaningful way. In the few cases where its activity has been assayed in discrete tissues, PLA2 activity is higher in acontia than tentacles (Nevalainen et al. 2004), suggesting a role in defense rather than in predation. Nonetheless, PLA2s are active in acrorhagi (Macrander et al. 2015a), attesting to a broad role for PLA2s in Actiniaria.

PLA2s and MAC/PF cytolysins are the only identified constituents of sea anemone venom that have a clear relationship to genes found in other taxa, including other Cnidaria (Nevalainen et al. 2004). Phylogenetic analysis of the PLA2s from sea anemones with those from other animals (Macrander et al. 2015a) indicates that the PLA2s of anemones have multiple origins within the larger gene family, rather than representing a single radiation. The presence of multiple copies from this gene family within a single species and the distribution of these on the tree points to within-species and within-lineage diversification of PLA2s in sea anemones.

Cysteine rich secretory proteins (CRiSP) play diverse roles in animals, including a role in venom in snakes. These are part of a larger gene family (CAP) whose members are critical to reproductive and immune function in vertebrates and critical to the organization and development of the nematocyst capsule (Balasubramanian et al. 2012). Genomic assays of *Nematostella vectensis* and studies of the nematocyst proteome detected genes with CRiSP and CAP domains, including some that could not be assigned to the structural proteins of nematocyst capsules (Moran et al. 2013). Similar molecules occur in the metridioidean sea anemone *Metridium senile* and the actinioidean *Heteractis magnifica*, in the scleractinian *Porites* and *Acropora*, and in the hydrozoan *Hydra* (Moran et al. 2013). Other cysteine-rich peptides include SCRiPs, smaller molecules that were first described from scleractinians and inferred to play a role in stress response and skeletalization. Sequences similar to SCRiPs occur in *Metridium senile* and in the actinioidean *Anemonia viridis* (Jouiaei et al. 2015). The toxicity of these is unknown, as is their affinity to those molecules in scleractinian corals.

Conclusions and Future Directions

Venoms of sea anemones differ from those of non-cnidarian animals in several respects: the distributed nature of venom in the cnidarian body, the great age of the lineage, and the reliance on lineage-specific genes for venoms. Perhaps unsurprisingly given these differences, the mode and tempo of evolution differs in sea anemones compared to that reported for, e.g., snakes, spiders, and cone snails. Unlike most other lineages from which venoms have been studied, the venom genes of sea anemones undergo concerted evolution (Moran et al. 2011) and

experience negative selection (Jouiaei et al. 2015) in addition to diversifying and positive selection. This difference may be a general property of venom genes in ancient lineages (e.g., Sunagar and Moran 2015) or may be an artifact of the way in which venom genes are studied in this lineage. Those molecules shared across major lineages conform more closely to the expectations of negative selection (see Jouiaei et al. 2015). Because studies of venom in sea anemones have largely been directed using the "search image" of a known molecule (Sunagar et al. 2016) and because the taxon sample of species surveyed is sparse in number and broad in phylogenetic diversity, it will be difficult to detect novel molecules or highly divergent ones, reinforcing the perception that there is low diversity within and across sea anemones in terms of their venom genes. Advancing our understanding of the evolution of venom in sea anemones will require focused study of lineages, tissues, and molecules. Evolutionary relatedness, function, and tissue of origin need to be considered. The strong correlation between toxin diversity and research effort suggests that taxonomic diversity will be key to the discovery of novel molecules. Nonetheless, lineage-specific differences in venom diversity clearly obtain with the anenthemonaean anemones generally having fewer kinds of toxins in their venom arsenal (Fig. 2). Although genomics and transcriptomics offer much potential for broad-based surveys of taxa and tissues, functional characterization, including studies of the biological role in natural systems, is critical to understanding whether and how these molecules have changed in light of sequence evolution and for clarifying the action of multifunctional molecules.

Cross-References

- ▶ Evolutionary Context of Venom in Animals
- ▶ Independent Origins of Scorpion Toxins Affecting Potassium and Sodium Channels
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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Revising the Role of Defense and Predation in Cone Snail Venom Evolution

6

Jutty Rajan Prashanth, Sebastien Dutertre, and Richard James Lewis

Abstract

Venoms are widely employed by numerous animals across disparate lineages for predation and defense. Among them, the deadly carnivorous cone snails are reputed for the potency of their venoms comprising small neurotoxic peptides known as conotoxins. Though a majority of cone snails prey on worms, some species prey on fish and other mollusks despite being slow movers. This remarkable prey diversification contributes to their evolutionary success. The origins of these dietary shifts have historically been explained based on the synergistic pharmacology of toxin classes. However, the recent discovery that some mollusk- and fish-hunting snails inject distinct defensive and predatory venoms has led to an alternative hypothesis where defense plays a pivotal role in the evolution of conotoxins and cone snails. This chapter provides an overview of cone snails and highlights recent advances in our understanding of conotoxin evolution.

Keywords

Cone snails • Conotoxins • Defense • Ecological release • Evolution

Note: This template intends to guide authors in writing a contribution for the handbook series *Toxinology*. As a reference work, please avoid first-person usage. Please also define all acronyms and abbreviations used in your contribution and avoid footnotes and endnotes. For further details, please refer to your author guidelines.

J.R. Prashanth (✉) • R.J. Lewis (✉)
Centre for Pain Research, Institute for Molecular Bioscience, The University of Queensland, St. Lucia/Brisbane, QLD, Australia
e-mail: p.juttyrajan@imb.uq.edu.au; r.lewis@imb.uq.edu.au

S. Dutertre (✉)
Institut des Biomolécules Max Mousseron (IBMM) – UMR 5247- Equipe Sciences Analytiques des Biomolécules et Modélisation Moléculaire, Université de Montpellier 2, Bat. 17, Montpellier, France
e-mail: sebastien.dutertre@umontpellier.fr; Sebastien.Dutertre@univ-montp2.fr

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_18

Contents

Introduction

Ecological interactions between life-forms have profoundly shaped their evolutionary histories (Dobzhansky 1973; Valen 1973). The nature of these interactions ranges from the mutually beneficial (mutualistic) to the competitive (competition for resources) and adversarial (predation, parasitism, etc.). Organisms across kingdoms have evolved numerous adaptations to gain advantages in such interactions (Dobzhansky 1973; Valen 1973). One such adaptation is the use of venom comprising a mixture of proteins and peptides secreted by a specialized organ that is injected through specialized injection mechanisms (Casewell et al. 2013; Lewis and Garcia 2003). Venoms have risen independently across many lineages for use in prey capture and defense. Some well-known venomous animals such as snakes, spiders, and scorpions inject venoms using their fangs or stingers into prospective prey to impair them, while others including most venomous fish and hymenopterans use their venoms in defense (Casewell et al. 2013).

Typically, venom peptides potently and specifically target a number of the receptors, enzymes, and ion channels that play key roles in important physiological processes, making these molecules highly efficient in their respective ecological roles, as well as providing promising drug leads (Lewis and Garcia 2003). Despite their infamy, venoms are an excellent source of novel compounds with the potential to treat a range of diseases. One example, captopril, a highly commercially successful ACE-inhibitor, was originally developed from a peptide isolated from the venom of the snake *Bothrops jararaca* (Prashanth et al. 2014). In addition to their medical potential, venom systems provide excellent opportunities to study evolutionary mechanisms such as protein neofunctionalization, and molecular and adaptive evolution. Venoms are hypothesized to be evolving in an "evolutionary arms race" (Dawkins and Krebs 1979) where increased resistance in prey catalyzes commensurate gains in potency and specificity of the toxins, and necessitates variations in overall venom composition (Casewell et al. 2013; Dawkins and Krebs 1979; Valen 1973). Indeed, venom composition within a species is adaptable

to local pressures, with well-characterized intraspecific variations in cone snail venoms a leading example (Jakubowski et al. 2005). The venoms of both snakes and cone snails exhibit variability across geographic regions in response to changes in diet (Chang et al. 2015; Daltry et al. 1996). Thus, selection pressure acting on venomous animals to efficiently capture prey can lead to increased prey specificity and potency of venoms (Pawlak et al. 2006). Several complementary genetic, transcriptional, and peptide level mechanisms are hypothesized to collectively facilitate the adaptive evolution of toxins and neofunctionalization to target new physiological receptors (Casewell et al. 2013). Though venoms appear to have adaptively evolved prey specificity, they are also used in defense, with snakebite associated with a large number of human fatalities (Kasturiratne et al. 2008). Similarly, some cone snail stings have also been associated with fatalities (Dutertre et al. 2014a). However, there is scant evidence about the role of defense in venom evolution (Casewell et al. 2013). Given defensive use of venoms against humans has prompted the multitude of venom-based drug discovery initiatives, combining these programs with an understanding with the evolution of venoms could better guide such efforts and also aid antivenom discovery – an intriguing twist on Dobzhansky's words – "Nothing in biology makes sense except in the light of evolution" (Dobzhansky 1973).

This chapter focuses on venom evolution in cone snails, slow-moving tropical snails whose deadly neurotoxic venoms are composed of small peptides called conotoxins. Conotoxins have proven a valuable source of molecular probes to dissect the pharmacology of receptors implicated in the pathophysiology of numerous neuronal diseases and disorders (Lewis et al. 2012). More importantly, a conotoxin has also been approved by the FDA to treat intractable pain and a number of other conotoxin-based drug leads are in various stages of clinical trials (Lewis et al. 2012; Prashanth et al. 2014). Uniquely, some cone snails inject distinct cocktails of defensive and predatory venoms facilitated by a venom gland adaptation allowing for localized expression and secretion of different toxins (Dutertre et al. 2014b). This also provides an opportunity to elucidate the role of defense in addition to predation on venom evolution. As stated above, a better understanding of venom evolution affords the opportunity to further rationalize drug discovery efforts for conotoxins with species-specific activity. The following sections provide a brief overview of cone snails and conotoxins, their classification and nomenclature, the approaches employed to study them, and aspects of their evolution.

Cone Snails and Conotoxins

Marine gastropods belonging to the family Conidae, which consists of more than 800 species, are among the most evolutionarily successful marine animals (Puillandre et al. 2015). Conidae comprises four genera, namely, *Conus*, *Conasprella*, *Profundiconus*, and *Californiconus*, with the genus *Conus* containing

~85 % of known species (Puillandre et al. 2015). *Conus* and *Conasprella* are further divided into 57 and 11 subgenera, respectively, based on molecular phylogenetic analysis (Puillandre et al. 2015). Cone snails have colonized tropical waters around the world using their venom for predation and defense. The wide geographical distribution and extended species diversity have been attributed to cone snails adapting to new environments by rapidly evolving their venom library, allowing them to capture newly encountered prey in novel environmental niches (Conticello et al. 2001; Duda and Palumbi 1999; Olivera et al. 2012). Indeed, cone snails have evolved to target a range of worm lineages and, surprisingly, mollusks and fish. Worm-hunting species (~80 % of Conidae) exhibit a wide variety of dietary patterns with some species employing a generalist approach, feeding on several different worm lineages, while others have specialized prey preferences (Duda et al. 2001; Kohn 1959; Leviten 1978). The array of biotic interactions encountered by Conidae is biochemically reflected in its venom composition (Olivera et al. 2012). Each cone snail species expresses a unique venom profile befitting its ecological requirements with initial estimates of the number of peptides per species ranging between 50 and 200 (Jones et al. 1996), though more advanced mass spectrometry methods have identified more than a thousand unique molecules (Biass et al. 2009; Dutertre et al. 2013; Lewis et al. 2012). Such an extensive reservoir is proposed to have evolved under natural selection pressure to maintain an efficient prey capture strategy, driving toxin diversification through aggressive mutations, gene duplications, and recombination (Conticello et al. 2001; Duda and Palumbi 1999; Olivera et al. 2012).

The toxins that make up cone snail venoms are called conotoxins. Conotoxins are primarily small disulfide-rich (containing two or more disulfide bonds) neuroactive peptides that predominantly range between 10 and 35 amino acids in length though longer peptides and proteins have also been discovered (Lewis et al. 2012). Conotoxins have a broad range of pharmacological targets comprising ion channels and neuronal receptors, many of which have been implicated in pain signaling pathways and the pathophysiology of neurodegenerative diseases such as Parkinson's and dementia (Lewis et al. 2012). Some prominent receptor targets include nicotinic acetylcholine receptors (nAChRs), voltage-gated calcium and sodium channels, G protein-coupled receptors (GPCRs), the norepinephrine transporter (NET), and N-methyl D-aspartate (NMDA) receptors (Lewis et al. 2012). Conotoxins are unusually potent, and potency is often allied with the ability to discriminate between receptor subtypes (Lewis et al. 2012). The small size and relative ease of synthesis combined with their exquisite pharmacological properties have made conotoxins an invaluable source of pharmacological probes and therapeutic candidates (Prashanth et al. 2012). In 2004, the FDA approved the first conotoxin-based drug, the calcium channel blocker ω-MVIIA (Prialt™) originally isolated from the venom of *Conus magus*, as an intrathecal analgesic for intractable pain, while several others are in clinical trials (Lewis et al. 2012; Olivera and Cruz 2001). Despite these advances, conotoxins remain a relatively untapped source of therapeutic candidates given the remarkable diversity they encompass, with estimates suggesting that just 0.1 % of the

conotoxin pool has been studied thus far (Lewis et al. 2012; Prashanth et al. 2012).

Conotoxin Transcription and the Processes Driving Diversification

Conotoxins are renowned for their hyperdiverse structure and pharmacology and the mechanisms facilitating this diversity at genetic, transcriptional, and the peptide level (Prashanth et al. 2014). Molluscan genomes are characterized by the predominance of repeat regions with preliminary genomic analysis of cone snails revealing a similar pattern for conotoxins (Barghi et al. 2015). Numerous conotoxin genes are seemingly punctuated by introns, some of which encompass repeat regions that presumably facilitate recombination and influence conotoxin diversification (Barghi et al. 2015). A number of unexpressed conotoxin genes across various superfamilies were also discovered from preliminary genome analysis that are likely differentially expressed to enable adaption to new environmental challenges (Barghi et al. 2015; Chang and Duda 2014). Differential expression of conotoxin genes also appears to facilitate the reported intraspecific diversity in venom peptide compositions (Chang and Duda 2014).

Conotoxins are initially transcribed and translated into a prepropeptide containing a hydrophobic signal peptide at the N-terminal end, a propeptide region, and the mature peptide at the C-terminal. Signal and propeptide regions are enzymatically cleaved, leaving the mature peptide to be folded into its tertiary structure by chaperone proteins and maturation is completed with the incorporation of posttranslational modifications (PTMs) (Fig. 1; Prashanth et al. 2012, 2014).

Cone snails are renowned for their combinatorial library-like strategy that diversifies their venom compositions, exhibiting both inter- and intraspecific variations in venom profiles (Abdel-Rahman et al. 2011; Davis et al. 2009). Variations in the venom injected by a single specimen have also been observed over time in *Conus consors* specimens milked in captivity. Though these injected venoms revealed an archetypal venom fingerprint, differences were observed in minor components between injections (Dutertre et al. 2010). Some injections comprised of an entirely different venom profile, though these may be defensive stings (Dutertre et al. 2010, 2014b).

Several genetic mechanisms are suggested to be driving the expansion of the conotoxin repertoire. These include gene duplication, recombination, and focal hypermutations within the mature peptide, which has an elevated rate of

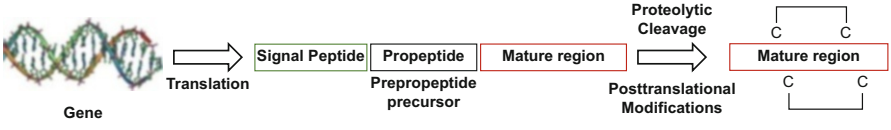

Fig. 1 The simplified conotoxin transcription pathway

non-synonymous mutations (mutations resulting in amino acid changes) (Barghi et al. 2015; Chang and Duda 2014; Conticello et al. 2001; Duda and Palumbi 1999; Olivera et al. 2012). In addition to genetic mechanisms, phenotypic mutations arising from "messy" transcriptional processing (Jin et al. 2013) and variable peptide processing in the form of truncations, posttranslational modifications and differential cleavages also greatly multiply the number of gene products, with more than 6000 peptides arising from just 105 conotoxin transcripts in the *Conus marmoreus* venom gland (Dutertre et al. 2013), further expanding conotoxin diversity. However, the majority of these variants are likely to be nonfunctional with functional versions upregulated and fixed in the genome over evolutionary time scales (Prashanth et al. 2016). The breadth of the conopeptide library likely provides the substrate for selection pressures to act on to improve selectivity at some receptors and attain activity at receptors that were not targeted previously, which in turn enables capture of new prey (Chang and Duda 2014; Olivera et al. 2012).

Classification of Conotoxins and Nomenclature

Three schemes are used to classify conotoxins. They are classified based on the gene superfamily, their structure and arrangement of cysteines in the mature peptide, and their pharmacological target. The gene superfamily of a conotoxin is assigned based on the signal peptide sequence of the conotoxin precursor and is denoted by a capitalized alphabet. Twenty-seven superfamilies have been accepted by the ConoServer thus far (Kaas et al. 2008) though recent transcriptome sequencing studies have identified many more novel superfamilies than were previously thought to exist (Prashanth et al. 2014). Whether these novel superfamilies are widespread across a number of species remains to be ascertained (Prashanth et al. 2014). A, O1, O2, M, and T are some of the most common conotoxin superfamilies, each with more than a 100 different precursors from various species deposited on the ConoServer (Kaas et al. 2008). The arrangement and connectivity of cysteines in the mature region influence the tertiary structure of the conotoxin. Hence, the arrangement of cysteines has been used to classify sequences into "cysteine frameworks" that are indicated using Roman numerals. Currently, there are 26 known frameworks, although as in the case of superfamilies, several novel frameworks have been discovered from transcriptome sequencing experiments, their prevalence across species is as yet unclear (Prashanth et al. 2014). Finally, based on their pharmacological targets, conotoxins are classified into families that are denoted by Greek alphabets, with 12 pharmacological families defined so far. The general template for naming, based on the original proposal of McIntosh et al. (1999), is illustrated with an example below (Fig. 2).

In the above example, the first Ca^{2+} channel blocker (ω-conotoxins) to be discovered and characterized from *Conus geographus* with the framework VI would take the name ω-GVIA. Conotoxins that have yet to be characterized pharmacologically are named similarly with the Roman number replaced by its equivalent Arabic number, and instead of an alphabet indicating the order in which the

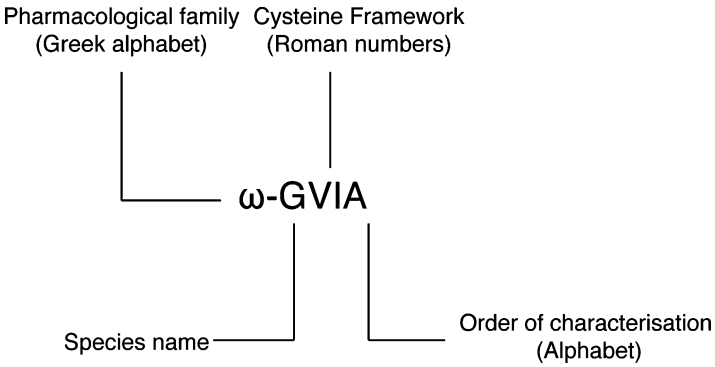

Fig. 2 Conotoxin nomenclature template using ω-GVIA as an example

conotoxins were classified, the equivalent Arabic number is used with the two numbers separated by a period. In the above example, before pharmacological characterization, ω-GVIA would have been termed G6.1.

While these classification schemes and nomenclature are applied to conotoxins, several "conopeptides," which are nominally defined as "cysteine-poor" peptides with one or no disulfide bonds with similarity to neurohormones, have also been discovered though they are less common than conotoxins and named differently (McIntosh et al. 1999; Prashanth et al. 2012). Non-conotoxin conopeptides are identified simply by their names, which are portmanteaus consisting of the name of the hormone/endogenous-signaling molecule to which the peptide shares greatest similarity, prefixed with the term "cono" and suffixed by the species name. Some examples of conopeptides include conopressins that are related to members of the vasopressin/oxytocin family, conopeptide Y/YY, which are similar to neuropeptide Y/YY, and conorfamide that resembles RF-amide peptides (Lewis et al. 2012). Based on the aforementioned nomenclature, the conopressin from *C. geographus* is called Conopressin-G.

The Integrated Venomics Approach Accelerating Conotoxin Discovery

Until recently, conotoxins were mostly isolated and characterized by a process called bioactivity-guided fractionation where venoms were screened for activity in bioassays and "hits" exhibiting activity were isolated by a series of fractionations punctuated by additional bioactivity screens (Prashanth et al. 2012, 2014). This resource-heavy approach uses a disproportionate amount of venom sample to characterize even a small number of peptides. Not only is this method taxing on venom resources, it is also time consuming and does not permit holistic studies of venom composition and properties which may be required to infer the ecological implications of some peptides being co-expressed/co-injected (Prashanth et al. 2012, 2014).

These drawbacks have rendered traditional bioactivity-guided methods cumbersome, particularly when trying to extract ecological and evolutionary information based on the composition of venoms. Rapid technological strides that have increased the availability and affordability of next-generation sequencing, as well as the development of advanced mass spectrometry techniques with exquisite sensitivity, and new bioinformatic tools to combine and analyze large volumes of data have seen the traditional approach gradually supplanted by sequence-driven approaches (Prashanth et al. 2012).

Apart from drastically reducing the time and resources required to characterize novel peptides, these sequence-driven approaches have greatly impacted whole venom and venom evolution studies by increasing the scale of these studies manifold and have also led to the discovery of several novel superfamilies (Prashanth et al. 2012, 2014). The integration of transcriptomic and proteomic data is an efficient way to analyze the composition of venom samples, and high-throughput bioassays can rapidly characterize the pharmacology of these peptides (Prashanth et al. 2012). The large volume of data generated by next-generation sequencing combined with proteomics also allows for specific evolutionary hypotheses to be tested.

Evolution of Cone Snails and Conotoxins

As mentioned earlier, cone snails consist of more than 800 species spread across the world inhabiting a range of environmental niches with a multitude of dietary preferences. Conidae are thought to have first appeared approximately 55 mya and subsequently diverged through two radiations that were separated by a period of extinction during the Lower Pliocene leading to the species diversity we observe today (Duda et al. 2001). While ancestral Conidae were likely worm hunters preying on errant polychaetes similar to the Turridae family from which they branched, modern cone snails prey on a range of errant and sedentary worms, mollusks, and fish (Duda et al. 2001). Each cone snail species possesses a unique mixture of venom peptides that are used to capture prey and in defense, with venom profiles reflecting the species' biotic interactions (Olivera et al. 2012). Even a conservative estimate of 100 conotoxins per species gives an estimate of 80,000 conotoxins, which is a tremendously diverse pool of toxins, though many of these conotoxins are likely to be homologous in activity.

Phylogenetic studies suggest that fish hunting evolved between one and three times, once or twice from worm-hunting cone snails that preyed on errant polychaetes, and once from sedentary polychaete-consuming species, while mollusk hunting evolved once from errant polychaete feeders (Duda et al. 2001). Eunicids remain the dietary preference for a majority of worm-hunting lineages that prey on errant polychaetes though some subgenera preferentially prey on nereids. Some species such as *Conus anemone* consume both eunicids and nereids, while some species such as *Conus arenatus* consume both errant and sedentary polychaetes (Duda et al. 2001). As opposed to the more generalist feeding behaviors within

worm-hunting Conidae discussed above, *C. leopardus* feeds exclusively on hemichordates (Duda et al. 2001) with its venom appearing to have undergone commensurate simplification (Remigio and Duda 2008). In keeping with this trend, increased dietary breadth in a *C. milliaris* population that underwent ecological release on Easter Island seems to have elicited a proportional expansion in its venom repertoire, presumably to allow capture of a wider variety of prey (Duda and Lee 2009). Furthermore, cone snail species spread across vast geographical areas with differing prey availability in each area also exhibit intraspecific variations in venom composition (Duda et al. 2009). Thus, the diet of cone snails and venom composition and evolution appear intertwined.

Traditional Views on the Origins of Prey Shifts in Conidae

The relationship between diet and venom leads to perhaps the most intriguing question regarding cone snail evolution and the origins of mollusk and fish hunting. The ability of cone snails to hunt fish is particularly surprising given snails move slowly. Despite their lack of mobility, piscivorous species have evolved two alternate mechanisms to capture prey. In the first, broadly called "hook-and-line," though variations of this strategy exist (Olivera et al. 2015), cone snails inject venom to rapidly immobilize fast-moving piscine prey (Fig. 3). This is the predominant feeding behavior among fish-hunting snails

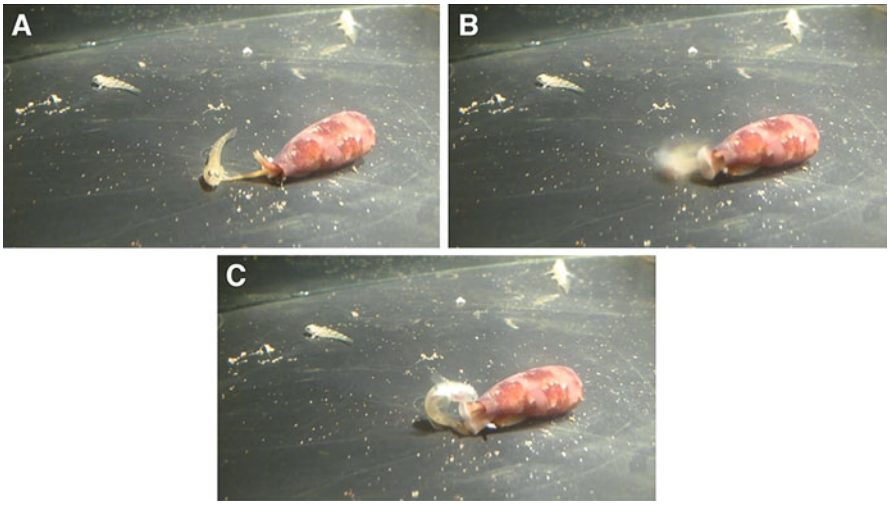

Fig. 3 The "hook-and-line" prey capture strategy by *Conus obscurus*. (**a**) *C. obscurus* approaches the fish with proboscis primed for stinging. (**b**) Snail stings the fish causing the fish to experience a massive excitotoxic shock resulting in rapid uncontrolled flinching before tetanic paralysis within seconds of injection. (**c**) Snail engulfs paralyzed fish

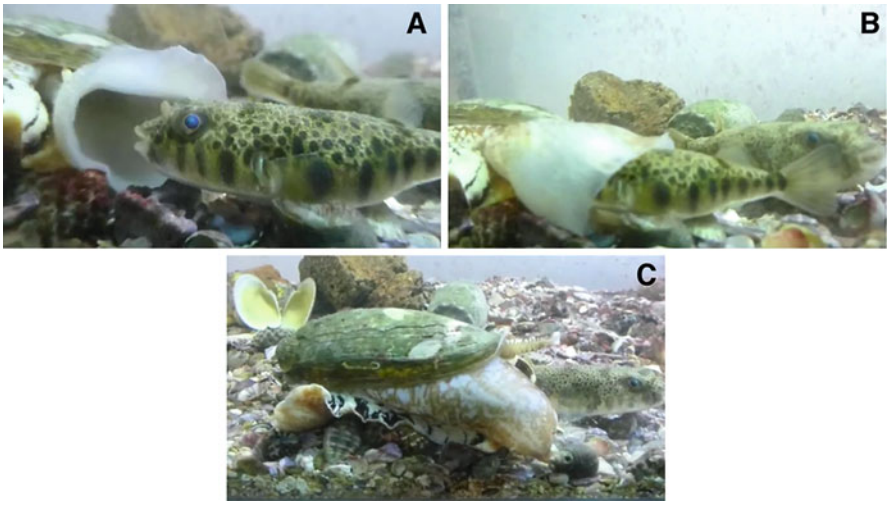

Fig. 4 The "net feeding" behavior of *Conus geographus*. (**a**) *C. geographus* secretes the "nirvana" cabal into the water causing the fish to become comatose. (**b**) The snail slowly engulfs the fish while it continues to remain dazed. (**c**) The fish is completely engulfed and is stung while inside the rostrum, presumably inducing paralysis to prevent escape as the fish briefly regained mobility

and presumably used by all of the piscivorous subgenera (Olivera et al. 2015). The second type called "net feeding" has only been reported in *C. geographus* and *C. tulipa* (subgenus *Gastridium*). Here, the cone snail opens its rostrum and secretes peptides into the water eliciting a "nirvana-like" state in the fish before engulfing them (Fig. 4; Olivera et al. 2015).

The molecular mechanisms reportedly underlying the two feeding behaviors are characterized by synergistically acting peptides referred to as "cabals." Hook-and-line hunters use two complementary cabals called "lightning-strike" and "motor" cabals. The lightning-strike cabal causes rapid tetanic paralysis, while the motor cabal induces flaccid paralysis by deactivating neurotransmission after tetanic paralysis has ensued (Olivera et al. 2015). The lightning-strike cabal is proposed to contain a peptide to delay the inactivation of Na^+ channels, usually a δ-conotoxin, and another to block shaker K^+ channels (Terlau et al. 1996). While δ-conotoxins appear to be used broadly by most if not all piscivorous cone snails, the choice of the K^+ channel blocker varies, with *Conus purpurascens* (*Chelyconus* subgenus) using a κ-conotoxin with the framework VII (Terlau et al. 1996), while *Conus striatus* (*Pionoconus* subgenus) is suggested to use a kunitz domain containing conkunitzin peptide for the same role, and *C. radiatus* (*Phasmoconus* subgenus) purportedly uses kRIIIK, a M-superfamily peptide with the framework III that is generally associated with Na^+ channel blockers (μ-conotoxins) (Olivera et al. 2015). Thus, the use of δ-conotoxins is widespread

in the lightning-strike cabal, while many of the K^+ channel blockers appear to be convergently recruited from a variety of structural folds. Following the lightning-strike cabal, the motor cabal, which consists of paralytic α-conotoxins (nAChR blockers), ω-conotoxins (Ca^{2+} channel blockers), and μ-conotoxins (Na^+ channel blockers), begins to systematically disable neurotransmission causing flaccid paralysis (Olivera et al. 1985; Terlau et al. 1996). The components of the motor cabal are independently paralytic and are widespread across piscivorous and molluscivorous cone snails (Olivera et al. 1985).

In contrast, the net feeding *C. geographus* and *C. tulipa* appear to have replaced the lightning-strike cabal with peptides that dampen the neuronal systems of fish such as contulakin-G and conanotkin-G that are both associated with inducing comatose symptoms in fish (Olivera and Cruz 2001). Recently, Conoinsulins that induce hypoglycemic shock in fish were also isolated from *C. geographus*, suggesting it may be another component of the "nirvana cabal" (Safavi-Hemami et al. 2015). This novel feeding behavior allows these Conidae to engulf several fish simultaneously aided by an extremely light shell and a large aperture, which in turn is presumed to allow these species to be more mobile and prey on schools of fish (Olivera et al. 2015). In addition, *C. geographus* and *C. tulipa* also express the motor cabal toxins described earlier, which are suggested to be occasionally injected to paralyze fish that have already been engulfed, likely to prevent escape (Olivera et al. 2015).

Based on the molecular pharmacology of these toxins and their combined physiological effects on fish, an evolutionary pathway to fish hunting from ancestral worm-hunting species was hypothesized (Olivera and Cruz 2001; Olivera et al. 2015). Briefly, it has been suggested that ancestral worm-hunting species originally evolved δ-conotoxins for predation. However, cone snails also use their venoms to stave off competitors and in defense. Hence, when a fish competing for the same prey approached, the ancestral Conidae are presumed to have injected it with a venom containing δ-conotoxins. It is suggested that δ-conotoxins that caused pain in fish apart from potently immobilizing worms were subsequently selected for. K^+ channel blockers that were recruited later by ancestral Conidae for predatory purposes now enabled rapid paralysis when used against competitor fish given δ-conotoxins were already established in the venom. Immobilization of injected fish likely provided opportunities for fish hunting before founder populations diverged, with some species eventually evolving into specialist fish hunters (Olivera et al. 2015). Thus, conservation in pharmacology across competitors, predators, and prey is predicted to have led to dietary shifts to include erstwhile piscine competitors and predators. Notably, this hypothesis does not adequately explain the evolution of mollusk hunting even though mollusks, including other cone snails, are also likely to act as competitors for targeted prey. No k-conotoxins have been found so far in mollusk-hunting species though some k-conotoxins have been found in worm-hunting snails (Lewis et al. 2012; Olivera et al. 2015), suggesting mollusk hunting may have followed an independent evolutionary trajectory.

Evolution of Distinct Predatory and Defensive Venoms: A New Hypothesis for Prey Shifts in Conidae

Though cone snails use their venoms primarily for prey capture, on occasion they are used to defend themselves against predators. Indeed, the venom of *C. geographus* is deadly and associated with numerous human fatalities accompanied by symptoms of paralysis consistent with the use of the "motor cabal" toxins, i.e., α-, μ-, and ω-conotoxins (Dutertre et al. 2014b; Fegan and Andresen 1997). Though the venom of *C. geographus* has been implicated in fatalities, stings from several other species have also been reported to cause severe symptoms suggesting that defensive use of venom is widespread across Conidae (Olivera and Cruz 2001; Olivera et al. 2015). The effects associated with cone snail envenomation were again attributed to the use of venom containing peptides that had evolved to target vertebrate prey and therefore more active on mammalian targets (Endean and Rudkin 1963; Olivera and Cruz 2001). However, the remarkable potency of *C. geographus*, which is a net feeder that rarely uses motor cabal peptides for prey capture, appears paradoxical since the motor cabal peptides in this species are presumably under relaxed selection pressure given their secondary role in predation. Yet *C. geographus* is the only species that is widely accepted to cause human fatalities (Dutertre et al. 2014a; Fegan and Andresen 1997).

This discrepancy maybe explained by the recent discovery that Conidae can alternatively inject distinct venom cocktails when provoked with predatory and defensive stimuli (Dutertre et al. 2014b). The motor cabal toxins that were originally hypothesized to be injected for predation were found to dominate the highly complex defensive venom of *C. geographus*. However, peptides purportedly part of the nirvana cabal were found to dominate the prey-evoked venom, which was much less paralytic than the defensive venom (Dutertre et al. 2014b). In contrast, motor cabal toxins dominated the defensive venom from different *C. geographus* specimens, accounting for the high lethality of the venom to humans (<0.05 mg/kg; Dutertre et al. 2014a). Furthermore, it appears *C. geographus* evolved a particularly aggressive defensive strategy, apparently trading off the protection afforded by a heavy shell for a highly complex and potent defensive venom that is readily deployed under duress. This stands in contrast to most Conidae that use their shells as the primary defensive mechanism with defensive injections employed only under exceedingly high threat (Dutertre et al. 2014b). Such a defensive envenomation strategy presumably coevolved with the novel net feeding strategy that allows multiple fish to be consumed simultaneously (Dutertre et al. 2014b; Olivera et al. 2015). The ability to readily switch between predatory and defensive venoms was also reported in *Conus marmoreus*, a mollusk-hunting cone snail, in which a majority of previously isolated mammalian active peptides were found to be major components in the defensive venom (Dutertre et al. 2014b). Defensive and predatory milkings were also collected from a number of other piscivorous and molluscivorous cone snails, while defensive stings were obtained from worm-hunting species, suggesting that this ability is widespread among Conidae (Fig. 5; Dutertre et al. 2014a, b).

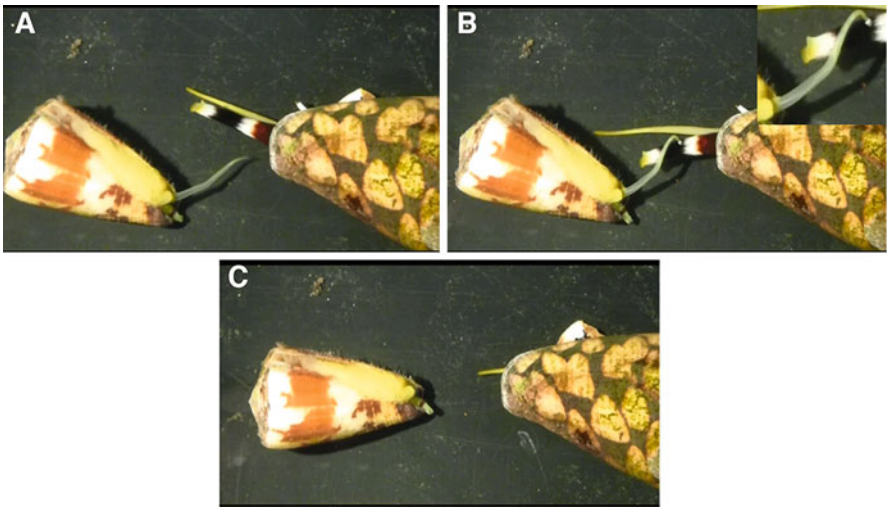

Fig. 5 Defensive envenomation by *Conus planorbis* against the predatory mollusk *Conus marmoreus*. (**a**) *C. planorbis* under threat from *C. marmoreus* extends its proboscis to defend itself against the mollusk-hunter *C. marmoreus*. (**b**) *C. planorbis* stings the predator on its siphon. The white fluid visible at the point of contact is the venom (see *inset*). (**c**) *C. marmoreus* withdraws its proboscis into the shell following the unsuccessful attempt to prey on *C. planorbis* due to its defensive envenomation against *C. marmoreus*

The spatiotemporal stimulus-dependent injection of venom is facilitated by compartmentalization of the venom gland (Dutertre et al. 2014b). Cone snails express venom peptides in a long tubular organ called the venom gland or duct, which culminates with the venom bulb at one end (proximal) and the proboscis at the other (distal). Glands of both *C. geographus* and *C. marmoreus* were sectioned with the "distal" section expressing predatory conotoxins and the "proximal" section of the gland expressing defensive venom peptides (Dutertre et al. 2014b). The target is stung using hypodermic needles-like radula through which the venom is injected for prey capture and/or defense (Fig. 6; Salisbury et al. 2010; Schulz et al. 2004).

Based on the above observations, Dutertre et al. have put forward an alternate hypothesis to better explain the evolution of mollusk and fish hunting. They suggest that the ancestral worm-hunting cone snail used a single venom cocktail, primarily for prey capture and secondarily for defense. However, in contrast to the previous hypothesis, some worm-hunting snails evolved a specialized venom gland that facilitated the injection of distinct defensive and predatory venoms. This adaptation was hypothesized to allow conotoxins to evolve separately under predatory and defensive selection regimes, with peptides originally evolved in worm-hunting cone snails for defense against predatory mollusks and fish (Dutertre et al. 2014b; Kohn 1959) being repurposed for mollusk and fish hunting, respectively (Dutertre et al. 2014b; Fig. 7).

While the aforementioned hypotheses help to better explain the striking diet diversification to mollusk and fish hunting, ~80 % of modern cone snails continue

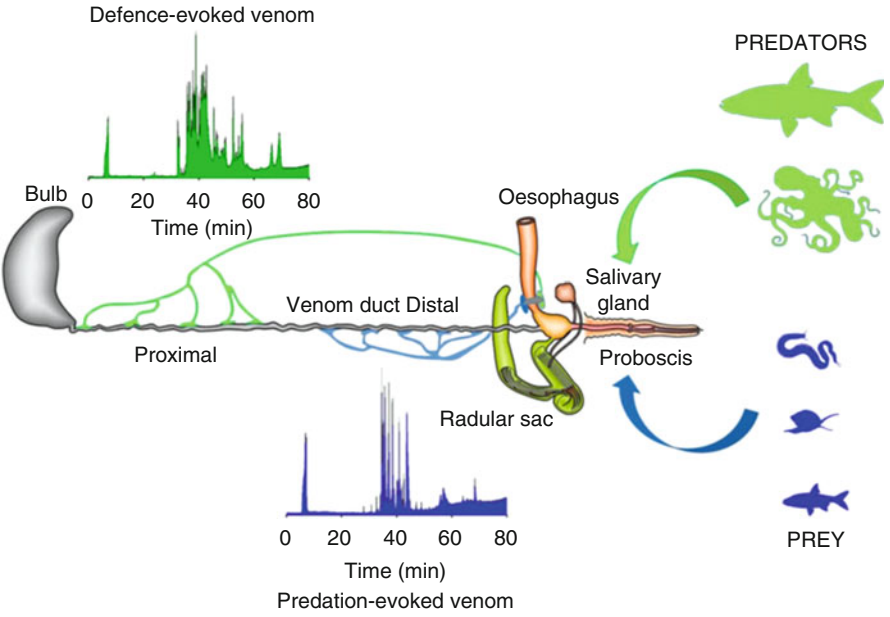

Fig. 6 The stimulus-dependent injection of venom by cone snails. (Adapted from Dutertre et al. (2015))

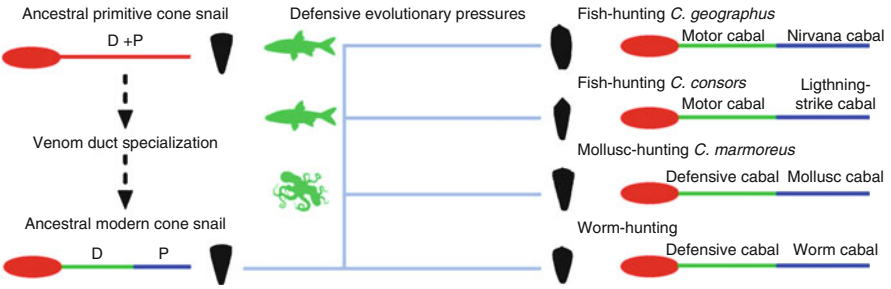

Fig. 7 An illustration of the "repurposed defense" hypothesis (Figure adapted from Dutertre et al. (2014b))

to prey on worms. More recent studies on worm-hunting cone snails are now starting to provide a more comprehensive understanding of the evolution of Conidae. Aman et al. isolated a δ-conotoxin from *Conus tessulatus*, a worm-hunting species that is phylogenetically closely related to fish-hunting subgenera. Behavioral observations of this species revealed that *C. tessulatus*, while primarily a worm hunter, can also opportunistically prey on fish. A homologous peptide was

also isolated from a closely related species, *C. eburneus* (Aman et al. 2015). A third δ-conotoxin SuVIA was isolated from *C. suturatus*, another member of the *Tesselliconus* subgenus (Jin et al. 2015a). Mass spectrometric studies revealed that expression of this peptide occurred primarily in the proximal section of the venom gland, which is associated with a defensive role, suggesting that these δ-conotoxins were likely originally evolved for defense against vertebrate predators including fish. Further, δ-SuVIA potently activates the human $Na_v1.7$ channel, an important part of the pain transmission pathways, thereby causing pain when defensively injected into vertebrate predators (Jin et al. 2015a). Together, these studies indicate that defensive δ-conotoxins from worm-hunting cone snails could have facilitated fish hunting, providing the first direct evidence of a link between defense and piscivory in Conidae, in a classic case of the hunter becoming the hunted (Jin et al. 2015a).

The role of defense on conotoxin evolution was also established by studying αD-conotoxins from a number of worm-hunting subgenera, predominantly the *Rhizoconus* subgenus (Prashanth et al. 2016). αD-conotoxins are dimeric nonparalytic inhibitors of nAChRs that appear to have evolved episodically for a defensive role in the *Rhizoconus* subgenus demonstrating that defense can play a role in the evolution of a toxin class. As with the δ-conotoxins, αD-conotoxins were also expressed mainly within the proximal venom gland of the *Rhizoconus* species where they have replaced the widely used α-conotoxins from the A-superfamily found in most if not all cone snail species surveyed to date (Prashanth et al. 2016). This integrated venomic study also showed that the compartmentalization of the venom gland that facilitated defensive and predatory venom injections in mollusk- and fish-hunting cone snails originally evolved in worm-hunting species, from where the other dietary classes arose. However, the aforementioned venom gland architecture is not conserved in all worm-hunting species. In *C. planorbis*, despite large variations in peptide expression along the venom gland, defensive venom peptides were more highly expressed in the central sections, in particular, the proximal central section (Jin et al. 2015b). Despite this difference, the defensive venom of *C. planorbis* was also highly potent at nAChRs although *C. planorbis* uses A-superfamily α-conotoxins unlike the αD-conotoxin-containing *Rhizoconus* subgenus. Why the compartmentalization of the *C. planorbis* venom gland is different to the other species studied is not yet clear. One possible explanation is that different worm-hunting species have evolved different venom gland compartmentalization with *C. planorbis* an example of this diversity. Another explanation is that ancestral cone snails used a single venom for defense and predation as hypothesized by Dutertre et al., with compartmentalization of the venom gland occurring over evolutionary time. Based on *C. planorbis*' early divergence as inferred from the phylogenetic reconstructions of Puillandre et al., it is possible that this species represents a transitionary venom gland from the earliest diverging species, where differences in venom expression along the gland were minimal, to modern mollusk- and fish-hunting species, where there are drastic differences between the different

sections of the venom gland (Dutertre et al. 2014b; Prashanth et al. 2016). Further studies into a wider array of species across different lineages of cone snails are required to fully uncover the evolutionary routes of Conidae, though these recent studies have begun to make some inroads.

Conclusion and Future Directions

This chapter highlights developments in our understanding of the ecological roles and evolution of conotoxins and cone snails. By injecting different defensive and predatory venoms, cone snails provide a unique opportunity to study the roles of each of these individually on the evolution of their venoms (Dutertre et al. 2014b). Indeed, recent studies have revealed an important role for defense in the evolution of Conidae (Prashanth et al. 2016). These discoveries have expanded our understanding of how prey shifts may have risen in this lineage. An early hypothesis explaining the origins of fish hunting based on the pivotal role of δ-conotoxins has been complemented by the discovery of their defensive role in worm-hunting Conidae that are closely related to fish-hunting species (Aman et al. 2015; Jin et al. 2015a). However, defensive venoms are typically complex and often contain peptides targeting Ca^{2+} channels and nAChRs (Dutertre et al. 2014b; Jin et al. 2015b; Prashanth et al. 2016). The roles of these peptides, if any, in facilitating prey diversification remain to be understood, and further research is required to unravel the evolutionary origins of these pharmacologically important peptides. Exploring the ancestry of these classes may also provide clues with regard to the evolution of mollusk hunting. Finally, little is known about the origins of conopeptides and conotoxins. The discovery of hormone-like venom peptides such as the insulin-like peptides in *C. geographus* (Safavi-Hemami et al. 2015) suggests a neurohormonal origin for some conopeptides. Nonetheless, the vast majority of conotoxins are disulfide-rich peptides with a rapid rate of evolution and small sizes that have diverged significantly enough to not have left a trace of their ancestry (Prashanth et al. 2016). Studying other cone snail organs may shed light on these fundamental questions about ancestral conotoxins. Finally, venomic studies on cone snails are beginning to shed further light on fundamental questions such as the origin of prey diversity (Aman et al. 2015; Jin et al. 2015a) and the role of defense in Conidae (Dutertre et al. 2014b; Prashanth et al. 2016). Though further study is required to fully understand these mechanisms, answering such evolutionary questions continues to offer the promise of rationalizing the discovery of novel conotoxins.

Cross-References

- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins
- ▶ Systematics and Evolution of the Conoidea

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Toxicity in Cephalopods

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Ira R. Cooke, Brooke Whitelaw, Mark Norman, Nikeisha Caruana, and Jan M. Strugnell

Abstract

Cephalopods are a morphologically diverse molluscan class that includes octopuses, cuttlefishes, squids, and nautiluses. The behavior, morphology, and sometimes aposematic appearance of coleoid cephalopods (octopuses, cuttlefishes, and squids) are highly suggestive of the widespread use of toxins for predation and/or defense. Many cephalopods use a combination of their parrot-like beak and/or toothed radula to inject venomous saliva, thought to be produced in the posterior salivary gland, into prey through a bite wound or a hole drilled into the shell. However, relatively few toxins have been studied to date from only a narrow range of cephalopods. Active components that have been identified from cephalopod posterior salivary gland extracts (or saliva) include neurotoxins such as tetrodotoxin (also found in body tissues), tachykinins and cephalotoxins, biogenic amines such as serotonin and octopamine, and a diverse range of enzymes including serine proteases, phospholipase A2, hyaluronidases, and chitinases. Coleoid cephalopods represent excellent candidates for biodiscovery, being taxonomically distinct from heavily studied venom-producing organisms, and

I.R. Cooke (✉)
Department of Biochemistry and Genetics, La Trobe Institute for Molecular Sciences, La Trobe University, Melbourne, VIC, Australia

Life Sciences Computation Centre, Victorian Life Sciences Computation Initiative, Melbourne, VIC, Australia
e-mail: I.Cooke@latrobe.edu.au

B. Whitelaw • N. Caruana • J.M. Strugnell
Department of Ecology, Environment and Evolution, La Trobe University, Melbourne, VIC, Australia
e-mail: blwhitelaw@students.latrobe.edu.au; njcaruana@students.latrobe.edu.au; J.Strugnell@latrobe.edu.au

M. Norman
Sciences, Museum Victoria, Carlton, VIC, Australia
e-mail: mnorman@museum.vic.gov.au

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_7

because their venoms appear to be complex mixtures of proteins and small molecules. Understanding the evolutionary history of toxicity in cephalopods remains a challenge, with many major taxa remaining unstudied and very little specific functional information available on most cephalopod toxins. The application of "omics" technologies to research on venoms and other toxic secretions ("venomics") is an important and powerful way of characterizing entire suites of proteinaceous toxins from pure venom or gland extracts in cephalopods and is likely to yield future insights into the evolution of toxicity in this class.

Keywords

Cephalopoda • octopus • squid • venom • coleoid • tachykinin • cephalotoxin

Contents

Introduction

The molluscan class Cephalopoda contains the octopuses, squids, cuttlefishes, and nautiluses. Although the greatest diversity of cephalopods is found in tropical shallow oceans, they occur in all marine habitats including the deep sea and polar regions. Cephalopods do not occur in freshwater environments. There are about 800 known species encompassing a large size range, from the giant squids (~14 m) to the pygmy squids (~2 cm). Most extant cephalopods do not possess an external shell, the exception to this being the nautiluses (subclass Nautiloidea). The remaining cephalopods (subclass Coleoidea) possess an internal shell, which has been reduced or lost in many cases throughout the course of evolution and thus they are vulnerable to predation. This lack of an external shell has likely driven the evolution of the remarkable ability of coleoids to change texture and color to blend in with their surroundings or, in some cases, to display warning colorations – potentially advertising their toxicity. In addition to the use of toxins in a defensive role, the behavior and feeding morphology of many coleoids is highly suggestive of the use of venom in subduing and/or externally digesting prey. The existence and use of toxins are so far unknown for nautiluses, and this review will therefore focus exclusively on coleoid taxa.

Coleoids are carnivorous, with many species, especially those that are benthic or demersal, specializing in hard-shelled prey such as bivalves, gastropods, crustaceans, and nautiluses (Pilson and Taylor 1961; Chichery and Chichery 1988; Saunders et al. 1991). The feeding apparatus of all coleoids is a small parrot-like beak, which would appear to be unsuitable for dealing with hard-shelled prey by crushing. Although brute force dismemberment is possible for smaller prey, it seems likely that venoms are often employed to at least weaken the prey, if not to kill it. This is well documented among many octopods, whereby the hard shell of the prey is penetrated by drilling a small hole through which a venom is injected (Grisley et al. 1996; Mather and Nixon 1995; Pilson and Taylor 1961; Runham et al. 1997; Saunders et al. 1991; Wodinsky 1969). Close observation of prey capture by cuttlefishes (Chichery and Chichery 1988) suggests that they also inject toxins through a small wound inflicted in a soft joint area of their prey (crabs). Likewise, the tiny (7–12 mm) Japanese pygmy squid (*Idiosepius paradoxus*) is capable of paralyzing shrimp much larger than itself (20–30 mm) within 1 min of capture (Kasugai et al. 2004) and also uses salivary secretions for external digestion.

The brightly colored, or otherwise striking, appearance of some octopus and squid species suggests that they may use toxins for defense (based on the aposematic warning colorations of diverse vertebrates and invertebrates). In some cases, such as in the brightly colored flamboyant cuttlefish (*Metasepia pfefferi*, see Fig. 1a), this striking coloration is coupled with diurnal activity in environments with high predator pressure (in contrast, many less colorful cephalopods are primarily nocturnal). The best-studied example of a cephalopod with a striking appearance is that of the blue-ringed octopuses (genus *Hapalochlaena*, see Fig. 1b), which flash iridescent blue markings when agitated (these cephalopods are also capable of excellent camouflage in order to stalk prey) (Norman 2000). *Hapalochlaena* species possess tetrodotoxin (TTX) in both the salivary glands and distributed throughout body tissues (Yotsu-Yamashita et al. 2007). The dramatic warning colorations and presence of the powerful neurotoxin throughout the body suggest more than a purely predatory role. Other examples include the striped pyjama squid (*Sepioloidea lineolata*) whose striking black-and-white striped color pattern suggests a warning to potential predators (Norman 2000). In addition, many coleoids that inhabit relatively shallow water environments produce ink that is released when attempting to evade a predator. Studies on the gastropod slugs known as sea hares (Aplysiomorpha), which also produce a form of ink, have discovered a complex cocktail of compounds in the ink that affect the behavior of predators such as lobsters (Derby 2007). Far less is known about the chemical composition and ecological role of cephalopod inks. Although some form of chemical defense in inks seems likely, this is yet to be demonstrated (Derby 2014; Wood et al. 2008).

These life history traits (hole drilling, aposematic coloration, lack of a hard protective shell, ink and slime production) suggest that cephalopods may possess a rich arsenal of toxic compounds in their venom and tissues. Nevertheless, the number of cephalopods for which any form of toxin research has been conducted remains low (fewer than 30 species), and the majority of these species appear in just

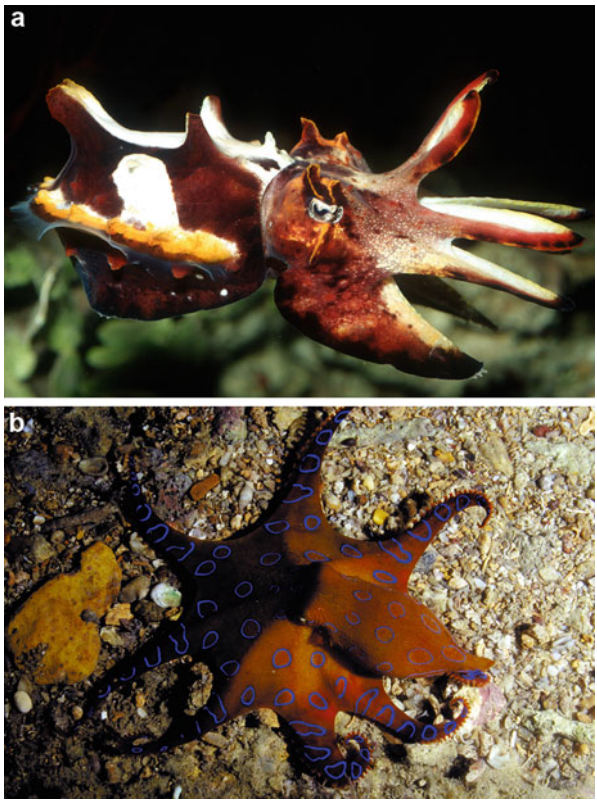

Fig. 1 Cephalopods with (possibly) aposematic coloration and displays. Photograph A is *Metasepia pfefferi* and B is *Hapalochlaena* sp. (Photograph A was taken by Roger Steene and B by Mark Norman)

four studies (Fry et al. 2009; Ruder et al. 2013; Ueda et al. 2008; Undheim et al. 2010). These four recent studies provide a glimpse into the diversity and potency of proteinaceous cephalopod toxins. Ueda et al. (2008) observed potent toxicity against crabs for posterior salivary gland (PSG) extracts of all three species of cuttlefish (*Sepia esculenta, Sepia lycidas, Sepia japonica*) studied and toxicity against both crabs and mice for PSG extracts of all three species of loliginid squids (Loliginidae) studied. This suggests that the use of potent venoms is widespread in cuttlefish and squids and that examination of a range of cephalopod taxa is likely to reveal different compounds (or variants) to account for varying target specificity. This is underscored by the studies by Ruder et al. (2013) and Fry et al. (2009) who provide a list of nine putative toxin protein families coded for in the transcriptomes of cephalopod posterior salivary glands.

Despite the high likelihood that cephalopods exhibit a diverse proteinaceous toxin arsenal, the number of well-characterized cephalopod toxins is extremely low. This is reflected in Fig. 2 which shows the number of proteins in the UniProt toxin database (verified toxins or components of venom) according to the taxonomic group from which the proteins were isolated. In this database, cephalopods, a

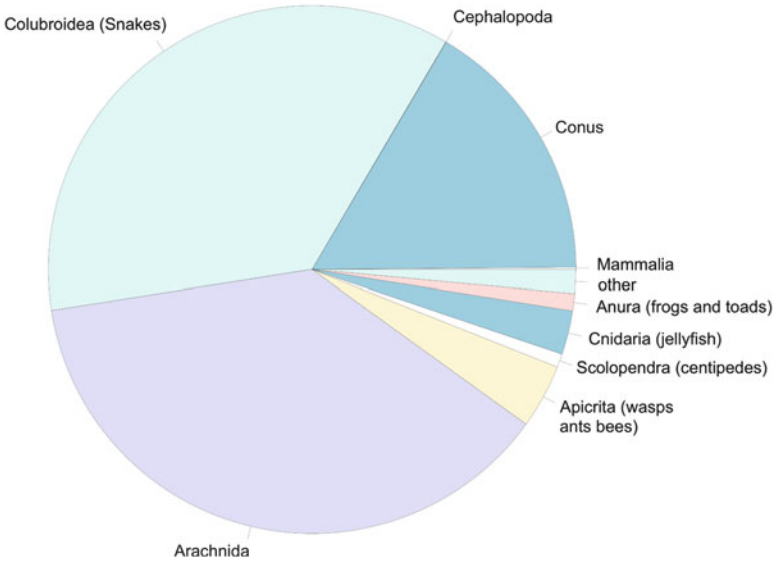

Fig. 2 Taxonomic distribution of 6,306 verified proteins in the UniProt toxin database. The database contains just a single cephalopod protein (SE-cephalotoxin). Note that at least one other cephalopod toxin (eledoisin) was erroneously missing from the UniProt toxin database at the time of writing

taxonomic group of some 800 species, are represented by just one protein (SE-cephalotoxin), whereas a single well-studied genus of gastropods (*Conus*) accounts for 1,022 entries.

Although recent venomics work provides strong evidence that cephalopod posterior salivary gland secretions are complex mixtures of many molecules (Cornet et al. 2014; Ruder et al. 2013), the effects of these compounds on potential prey (or predators) have not been established in most cases. In this review, we first focus on the most studied classes of molecules, including small molecules such as tetrodotoxin (TTX) and biogenic amines, as well as proteins such as cephalotoxin, chitinases, and peptidases. Later sections of the review summarize current knowledge on the evolution of toxicity in cephalopods and technological developments including the potential insights to be gained from venomics studies.

Since cephalopods produce a range of potentially toxic substances (salivary venom, ink, body tissues), it is important to clarify our use of the terms venom, venomics, toxin, and toxic. In this review we use the term venom to refer to substances injected via a bite or drill hole (i.e., salivary secretions) but accept that the term "venomics" can sometimes be applied to studies of other toxic secretions. We use the term toxin to refer to specific molecules that confer toxicity and emphasize that toxins may be contained within various tissues in the cephalopod body or secreted as part of venoms or inks. All substances that contain toxins are referred to as toxic.

Significant Toxin Classes in Cephalopods

Tetrodotoxin

Tetrodotoxin (TTX) is an extremely potent low molecular weight neurotoxin. In cephalopods, TTX is known to be present in members of the genus *Hapalochlaena* (blue-ringed octopuses). Many studies have focused their attention on the genus *Hapalochlaena* due to the immediate threat it poses to humans. The first documented fatal envenomation from a blue-ringed octopus occurred in 1954 in Darwin, Northern Territory, Australia (Jacups and Currie 2008). Studies into the posterior salivary gland (PSG) of *H. maculosa* identified the fatal component as maculotoxin (Crone et al. 1976), which was later revised to TTX (Sheumack et al. 1978). A similar toxin to TTX was isolated from *H. maculosa* called hapalotoxin, distinguishable from TTX due to its higher polarity (Savage and Howden 1977).

Aside from cephalopods, TTX also occurs in a diverse range of other organisms including fishes, amphibians, arthropods, nemerteans, flatworms, arrow worms, echinoderms, and other molluscs. The origins of tetrodotoxin are not fully understood, but its sporadic appearance across unrelated taxa suggests that it is either ingested or produced by bacterial symbionts (Chau et al. 2011). Studies on puffer fish have shown that captive animals lose toxicity over time if fed a non-TTX containing diet (Noguchi et al. 2006), which suggests accumulation via ingestion. However, the newt, *Taricha granulosa*, is able to maintain and even increase TTX levels on a non-TTX containing diet (Hanifin et al. 2002), which suggests that it is produced by symbionts or even endogenously by the newts themselves. Studies on puffer fish (Yu et al. 2004; Wu et al. 2005) have identified TTX-producing bacteria from the tissues of TTX-bearing host species. TTX in blue-ringed octopuses is thought to be bacterial in origin, but it is not known whether these bacteria are housed within a particular organ, spread throughout the body, or ingested. Hwang et al. (1989) cultured 22 strains from the tissues of *H. maculosus*, [sic] of which 16 were shown to produce TTX. TTX-producing strains were found in cultures made from the posterior salivary gland, tentacle, intestine, and ink sac and were identified as species of *Vibrio*, *Alteromonas*, *Pseudomonas*, and *Bacillus*.

Blue-ringed octopus are one group of only a small number of animals believed to use toxins for defense as well as to subdue prey. Evidence for a predatory role comes from the presence of TTX in the posterior salivary glands, which are the usual venom-producing organs in octopods, but its efficacy against typical prey has not been quantified (Williams 2010). Evidence for a defensive role comes from the characteristic iridescent blue warning rings that the animal displays when distressed, as well as from the relative distribution of TTX across tissues, eggs, and larvae. This intraorganismal distribution of TTX varies depending on species and geographic location (Hwang et al. 1989; Williams and Caldwell 2009). For example, Hwang et al. (1989) examined the relative concentrations of TTX in the posterior salivary glands versus other soft tissues, for two specimens collected at different locations. In one specimen (Cebu Island, Philippines), they found that the majority of total TTX was located in the soft tissues, with only around a quarter of total TTX being located

in the PSG. In contrast, a specimen from Izu Peninsula (Japan) had concentrated almost all TTX in its posterior salivary gland. This suggests that the relative role (defense or predation) of TTX in blue-ringed octopus may vary between individuals or subpopulations. Further evidence for a defensive role for TTX comes from Yotsu-Yamashita et al. (2007), who examined six specimens from South Australia and found much higher levels of TTX in bulk body tissues (arms, cephalothorax, abdomen) than in the posterior salivary gland. Williams et al. (2011) found that while *H. maculosa* larvae contained TTX, it was not at a sufficient concentration to intoxicate or deter predators in controlled feeding experiments. Interestingly, they found that *H. maculosa* larvae were indeed distasteful to predators but speculate that this is due to some compound other than TTX, since TTX-spiked control items were not distasteful to representative fish or stomatopod predators.

Utilization of a toxin necessarily requires an organism to also contain these highly detrimental proteins or compounds. As a result, the organism requires a degree of resistance to these toxic elements (Flachsenberger and Kerr 1985). The tolerance of one *Hapalochlaena* species (most likely *H. maculosa* based on the collection site) was examined by Flachsenberger and Kerr (1985), who found no negative effects when the specimen was injected with high doses of purified TTX or salivary extract. This suggests that any predatory or defensive function of *Hapalochlaena* venom is not directed at others of the same genus. In contrast, the male platypus utilizes its venom almost exclusively for intraspecies competition (Wong et al. 2012).

Cephalotoxin

The term cephalotoxin was first used by Ghiretti (1959) to describe a protein purified from the posterior salivary gland of *Sepia officinalis*. The defining characteristic of this protein was its ability to induce complete paralysis in crabs, a phenomenon that had previously been observed for whole octopus saliva as early as 1897 (Krause 1897). Later work by Ghiretti (1960), McDonald and Cottrell (1972), Songdahl and Shapiro (1974), and Cariello and Zanetti (1977) isolated protein fractions with similar neurotoxic effects from the common octopus (*Octopus vulgaris*), the curled octopus (*Eledone cirrhosa*), and the giant Pacific octopus (*Octopus dofleini*, now *Enteroctopus dofleini*). Although all of the proteins in these early works have been referred to as cephalotoxin, no sequencing or other detailed molecular characterization was performed, and it is not clear that they were homologues. Indeed, Songdahl and Shapiro (1974) noted that the molecular weight of their protein (23 kDa) was very different from the protein studied by McDonald and Cottrell (30–70 kDa) and suggested that cephalotoxins may be a diverse group.

Much more recently, Ueda et al. (2008) performed the first direct protein sequencing on a compound with the neurotoxic effects characteristic of cephalotoxin. This protein, purified from the posterior salivary glands of the golden cuttlefish (*Sepia esculenta*), was named SE-cephalotoxin and is one of only a small number of cephalopod toxins that have been purified and sequenced. At 1,052 amino acids, it is exceptionally large for a venom protein. Currently, the Tox-Prot database of over

6,000 proteins contains just 14 longer sequences and has a median length of 79 amino acids (http://www.uniprot.org/program/Toxins). Other features of the protein sequence are the presence of a signal peptide, pro-peptide, and multiple cysteine-rich regions, all of which are commonly observed in other venom proteins (Ueda et al. 2008). It is likely that SE-cephalotoxin is indeed a homologue of at least some of the proteins previously called cephalotoxin. It is highly glycosylated, which is consistent with observations made by Cariello and Zanetti (1977) on cephalotoxins isolated from the *O. vulgaris*.

The evolutionary origin of cephalotoxin remains unclear as very few homologues have been sequenced. SE-cephalotoxin homologous sequences have been found in *Sepia officinalis* (Cornet et al. 2014), Hawaiian bobtail squid (*Euprymna scolopes*) (Collins et al. 2012), the coral *Acropora millepora* (Ramos-Silva et al. 2013), and most recently the Australian ghost shark (*Callorhinchus milii*) (Venkatesh et al. 2014). In addition, Ruder et al. (2013) sequenced cephalotoxin homologues in the broadclub cuttlefish (*Sepia latimanus*), the pharaoh cuttlefish (*Sepia pharaonis*), and the southern reef squid (*Sepioteuthis australis*) but did not publish the assembled sequences or present a phylogenetic analysis for cephalotoxin. The existence of homologues in the coral and ghost shark genomes indicates an ancient (pre-molluscan) origin for the protein, and functional work (Collins et al. 2012) indicates that within *Euprymna scolopes*, the protein plays an important role in the organism's immune system that is possibly unrelated to its use as a venom.

Tachykinins

Tachykinins comprise a large family of highly conserved neurotransmitters found in vertebrates and invertebrates including cephalopods. They participate in both the peripheral and central nervous systems via afferent and efferent pathways, being involved in smooth muscle contraction, peripheral sensing, and neurogenic inflammation (Khawaja and Rogers 1996). Tachykinin receptor proteins (TKRP) mediate the effects of tachykinins via specific binding between each tachykinin and its associated receptor.

The first peptide toxin to be isolated and sequenced from cephalopod venom was the tachykinin eledoisin (Erspamer and Anastasi 1962), from the musky and curled octopuses (*Eledone moschata* and *Eledone cirrhosa*, respectively). This followed the discovery of substance P, a mammalian tachykinin in 1931 (von Euler and Gaddum 1931), and it was soon realized that these molecules were structurally and pharmacologically similar, being part of a large family widespread across many taxa (Khawaja and Rogers 1996). Eledoisin is present in the salivary glands of octopods from the genus *Eledone* (Anastasi and Erspamer 1963; Erspamer and Anastasi 1962). It induces hypotension and contraction of gut muscles in vertebrate (dog and guinea pig) assays (Anastasi and Erspamer 1962) but its effects on invertebrates have not been assayed.

More recent studies on *O. vulgaris* have revealed considerable additional detail about the role of tachykinins in octopods. Kanda et al. (2003) isolated two

tachykinins from the posterior salivary glands of *O. vulgaris* (Oct-Tk-I, Oct-TK-II), and subsequently, Kanda et al. (2007) detected a total of seven tachykinin-related peptides in *O. vulgaris* brain tissue. The first octopod tachykinin receptor (Oct-TKRPR) was identified by Kanda et al. (2007) from heart tissue of *O. vulgaris*, and it was found that this receptor is not responsive to tachykinins expressed in salivary glands (Oct-Tk-I), but that it is responsive to tachykinin-related peptides expressed in brain tissue (Oct-TKRP's I–IV). This suggests that production of tachykinins in saliva is likely to be specifically related to venom production rather than participating in the octopus' endogenous neurologic pathways.

Tachykinins and tachykinin-related peptides are usually small (approx 10–150 amino acids) and are often produced by cleavage of a larger precursor protein. It has been noted that vertebrate tachykinins often possess a motif of the form FXGLM, whereas in invertebrates it is FXGXR. A study by Ikeda et al. (1999) showed that the C-terminal residue within this motif (M for vertebrates, R for invertebrates) was crucial to the potency of neuromuscular effects for a tachykinin from an echiuroid worm and one of mammalian origin. In particular, they assayed invertebrate and vertebrate tachykinins against invertebrate and vertebrate assays and found that substitution of the C-terminal residue (R to M for invert tachykinin and M to R for vertebrate) could induce a switch in assay specificity from invertebrate to vertebrate and vice versa. Interestingly, tachykinins from the *O. vulgaris* posterior salivary gland and Eledoisin (found in posterior salivary glands of *Eledone*) all possess the vertebrate motif (FXGLM) despite the fact that their dominant prey items are invertebrates. Ruder et al. (2013) assayed the relative activity of three octopus tachykinins (two from *O. vulgaris* and one from *Octopus kaurna*), all of which had an FX[SG]LM motif using vertebrate and invertebrate assays. They found that all peptides elicited a response from both assays and that their relative potency was the same on invertebrate and vertebrate tissues. These results and others (e.g., Poels et al 2009) point to a deficiency in our current understanding of the differences between vertebrate and invertebrate tachykinins and their receptors, and that vertebrate vs invertebrate tachykinin specificity cannot simply be induced by analysis of the tachykinin motif amino acid sequence alone.

Chitinases, Peptidases, and Other Degradative Enzymes

Many venomous animals include degradative enzymes as components of venom and which may be classed as toxins due to their ability to cause cell or tissue damage or even neurotoxic effects (Kini 2003). These often target specific structurally important molecules whose degradation may have anticoagulant effects, leading to increased tissue permeability, cell lysis, or hemorrhage (Gutierrez and Rucavado 2000; Kini 2003; Kang et al. 2011; Wong and Belov 2012). This in turn can enhance the spread of other venom components and/or hasten the immobilization or death of the victim. It is likely that cephalopod venoms include molecules to perform these functions, but little is known about them. Transcript sequences from several major venom enzyme classes have been identified in cephalopod posterior salivary gland

extracts (Fry et al. 2009; Ruder et al. 2013) including hyaluronidase (*H. maculosa*, *O. cyanea*), serine peptidases (probably ubiquitous), and phospholipase A2 (*Loliolus, Sepia, Sepiotheuthis*). In addition, whole PSG extracts of four Antarctic octopus species (*Adelieledone polymorpha*, *Megaleledone setebos*, *Pareledone aequipapillae*, and *Pareledone turqueti*) have been assayed for alkaline phosphatase, acetylcholinesterase, phospholipase A2, hemolytic, and proteolytic activities (Undheim et al. 2010) with three species showing some activity in all assays. That these degradative enzymes are important components of cephalopod venoms is suggested by the fact that they have undergone diversification (in the case of serine proteases) within the cephalopod lineage (Fry et al. 2009) and have acquired adaptations to cold temperatures within Antarctic species (Undheim et al. 2010).

Chitinases and peptidases appear to be a ubiquitous component of cephalopod posterior salivary gland extracts, being found in all species for which transcriptomic sequencing has been performed (Fry et al. 2009; Ruder et al. 2013; Cornet et al. 2014), as well as being identified in numerous other species via bioassays (Grisley and Boyle 1987; Undheim et al. 2010; Grisley 1993) or by purification and direct sequencing (Ogino et al. 2014). As components of venom, they are likely to cause considerable damage to prey, breaking down muscle or connective tissue and/or assisting with cephalopod hole boring into the exoskeletons of crustaceans. A key role for chitinases, peptidases, and other digestive enzymes in cephalopod venom is likely to be external digestion. The cephalopod body plan includes a very small mouth and a narrow esophagus that passes through the doughnut-shaped brain, thereby placing strong constraints on the size of food particles that can be ingested. External digestion is particularly well documented for octopus, where the injection of salivary secretions greatly increases the ease with which crustacean prey can be dismembered (Grisley and Boyle 1987; Nixon 1984). By devising a specific bioassay for detachment of crab muscle from carapace, Grisley and Boyle (1987) were able to attribute this activity to a proteolytic enzyme contained in milked salivary extracts from *Eledone cirrhosa*.

Amines

Amines are low molecular weight organic compounds that are ubiquitously found in biological systems. Many amines are neurotransmitters, including epinephrine, norepinephrine, dopamine, serotonin, and histamine. They are frequently encountered as components of invertebrate venoms, and, while they are often responsible for producing an acute pain response (Welsh 1964), they are not often the cause of more serious effects such as paralysis or death (Welsh 1964).

Early work on cephalopod venoms identified a substance with the ability to induce smooth muscle contractions in mammals from posterior salivary gland extracts of *O. vulgaris*. This substance was then named enteramine, but in 1952 (Erspamer 1952) it was found to be identical to serotonin, a substance that had been studied independently by another group of researchers working on mammalian blood (Whitaker-Azmitia 1999). In addition to identifying enteramine/serotonin,

Erspamer (1952) also discovered octopamine from the posterior salivary gland extracts of *O. vulgaris*. The effects of octopamine in invertebrates and in vertebrates have been extensively studied (David and Coulon 1985). It acts as a neurotransmitter, neurohormone, and neuromodulator with effects on both the central and peripheral nervous systems (David and Coulon 1985). It has been shown that both octopamine and serotonin elicit a neurophysiological response from crustaceans (Livingstone et al. 1980), but because these molecules are so ubiquitous in biological systems, it is unclear whether their presence in posterior salivary glands indicates an active role in octopus venom itself.

Taxonomic Coverage and the Evolution of Toxicity in Cephalopods

The evolutionary history of toxicity in cephalopods remains relatively unknown, and in order to better understand it, two major challenges need to be overcome. The first is poor taxonomic coverage. Several taxonomic groups have received very little (e.g., cirrate octopuses [Cirrata], vampire squid [Vampyroteuthidae]) or no (e.g., argonauts, blanket octopus [Argonautoidea], bottletail squids [Sepiadariidae]) investigation. Also, relatively few species of some speciose groups have been investigated. For example, around six species from the family Sepiidae (cuttlefishes) have been investigated to date, yet the family is known to contain around 100 species. Similarly there are over 300 species known from the super family Octopodoidea, Strugnell et al. (2013), but only ~7 species have been investigated at the present time. Only a single species (*Todarodes pacificus*) from the order Oegopsida has been studied, yet at least 250 species are known to belong to the order. Therefore, we cannot be certain that the toxins identified to be present within those species studied are "representative" of the broader taxonomic group.

The second challenge is that almost no cephalopod-specific functional information exists regarding the molecular components of venoms. This means that although we may be able to identify protein variants that are specific to cephalopods, or perhaps to a particular cephalopod clade, we are limited in our ability to draw links with feeding behaviors or life history traits that would explain these evolutionary events. In the absence of cephalopod-specific functional information (e.g., studies on the specific effects of pure venom fractions), the best that can be achieved is to infer function based on sequence similarity with venom components from well-studied species such as cone snails, snakes, and arachnids. This is problematic because some families of venom proteins (e.g., PLA2, Kini 2003) include many variants that have evolved diverse and highly specific functions that cannot readily be determined from the amino acid sequence alone. Furthermore, since all venomics studies on cephalopods to date have used posterior salivary gland extracts as a proxy for venom, it is sometimes difficult to rule out the possibility that a putative venom component is in fact merely part of the salivary gland apparatus and not present in the actual saliva produced.

Several recent studies have made progress toward addressing these challenges and have provided the first significant insights into toxin evolution in cephalopods. A study by Ueda et al. (2008) assayed posterior salivary gland extracts from three cuttlefish species (*Sepia*), three loliginid squid species (*Loligo, Sepioteuthis*), and one oegopsid squid species (*Todarodes pacificus*) for lethal activity against mice and crabs (*Potamon dehaani*). They found that extracts from all cuttlefish species were most potent against crabs but had no activity against mice, whereas all the loliginid squids exhibited some potency against both mice and crabs, and the oegopsid squid had no lethal activity in either assay. This finding suggests that *Sepia* and loliginid squids possess specialized toxins to suit their preferred prey, with loliginids consuming a greater proportion of vertebrates (fish) than *Sepia* whose main diet is crustaceans (Hanlon and Messenger 1996). The complete lack of lethal activity from PSG extracts of *Todarodes pacificus* is also interesting because this species is from a different taxonomic group (order Oegopsida), contained within the Decapodiformes, from the six toxic species studied (which belong to the order Myopsida and family Sepiidae). Another recent study that used assays on whole PSG extracts to demonstrate toxin evolution was that of Undheim et al. (2010). They provide evidence that some Antarctic octopods have evolved cold-adapted enzymes as part of their venom. In particular, they showed that alkaline phosphatase activity was cold adapted (higher activity at 0 °C than at 37 °C) in all species and proteolytic activity was also cold adapted in three of four species tested.

Recently, two studies (Fry et al. 2009; Ruder et al. 2013) have specifically attempted to gain insights into toxin evolution in cephalopods by applying high-throughput transcriptome sequencing to posterior salivary gland extracts across ten cephalopod species from major octopod and decapod groups. These offer the first molecular phylogenetic insights into toxin evolution in these taxa, in addition to providing a broad overview of the protein composition of cephalopod venoms. Of particular interest is the finding by Fry et al. (2009) (later expanded upon by Ruder et al. (2013)) that a large number of S1 peptidases exist in the venoms of all ten species studied. At least for *H. maculosa*, *O. kaurna*, and *S. latimanus*, these molecules are collectively distinct from non-cephalopod taxa while being present throughout the cephalopod clade (Fry et al. 2009). Fry et al. (2009) argue that at least four successive gene duplication events have occurred prior to the divergence of the decapodiform and octopodiform lineages (revised to six events by Ruder et al. 2013) and that this suggests an ancient origin of the posterior salivary gland organ in cephalopods and, by extension, its use in venom production. In addition, Ruder et al. (2013) applied site-specific selection analyses to serine proteases and pacifastins. They found that although most sites were under negative selection, 26 serine protease sites and three pacifastin sites were under positive selection, with the majority of positively selected serine protease sites likely to be at the surface of the folded protein. Since changes at the protein surface are most likely to affect receptor binding and/or enzymatic function, positive selection at these sites agrees with a model for toxin evolution in which genes are duplicated and then one of the copies takes on a role as a toxin (Wong and Belov 2012). Given that venom proteins

are injected into a foreign victim, they are likely be subject to some positive selection in order to optimize efficiency against novel receptors or substrates.

New Technologies and Shifting Emphasis

Over the past 50 years, technologies for separating, assaying, and characterizing venom extracts and toxin molecules have become more sensitive and are providing increasing quantities of data. Constrained by older technologies, early studies tended to focus on a small number of abundant species (available commercially), used large volumes of starting material, and attempted to isolate a single molecule for detailed study. For example, Erspamer and Anastasi (1962) used salivary glands from 10,000 *Eledone* individuals in order to characterize the tachykinin peptide, eledoisin, and 30,000 *O. vulgaris* individuals were used by Erspamer and Asero (1953) to isolate enteramine. Such large quantities of material are astonishing by the standards of most modern studies where less than ten individual animals is the norm (e.g. Ruder et al. 2013; Ueda et al. 2008; Undheim et al. 2010). This trend toward the use of fewer animals is undoubtedly a positive one as it enables study of less abundant species, is significantly cheaper, and is more ethically sound.

Despite using reduced sample volumes, modern studies are able to generate vastly more data than ever due to the adoption of modern "omics" technologies. These allow whole genomes and transcriptomes to be sequenced via next-generation nucleic acid sequencing technology and for whole proteome surveys to be conducted using mass spectrometry. Collectively termed "venomics" (Escoubas and King 2009), these techniques allow data to be obtained on whole suites of molecules (e.g., whole animal or whole tissue), at low cost, from a small (milligram to gram) quantity of sample. Techniques related to venomics (indicated by arrows l, m, n, and o in Fig. 3) have so far seen very little application to cephalopod venom research. Notable exceptions are the studies by Fry et al. (2009) and Ruder et al. (2013), which performed transcriptome sequencing on posterior salivary gland (PSG) extracts of several species, and the study of Cornet et al. (2014), which performed both transcriptomic and proteomic analyses on PSG of *S. officinalis*. These studies identified numerous transcripts with homology to venom proteins observed in other taxa (snakes, arachnids, and cone snails) including CAP (CRiSP/Allergen/PR-1) proteins, carboxypeptidases, chitinases, hyaluronidases, pacifastins, phospholipase A2 proteins, SE-cephalotoxin, and serine proteases (Table 1).

Notable missing lines of inquiry from Fig. 3 include those corresponding to direct protein analysis, for example, by mass spectrometry-based proteomics, and particularly analysis of venom or pure toxic fractions. These are important because venom proteins are often posttranslationally modified (Buczek et al. 2005) and because transcripts may be expressed in venom gland tissue without necessarily being translated to protein and/or secreted into the mature venom. Posttranslational modifications can include modifications to the amino acid sequence (e.g., cleavage of pro-peptides, signal peptides, etc.) and site-specific modifications (Buczek et al. 2005; Kapono et al. 2013).

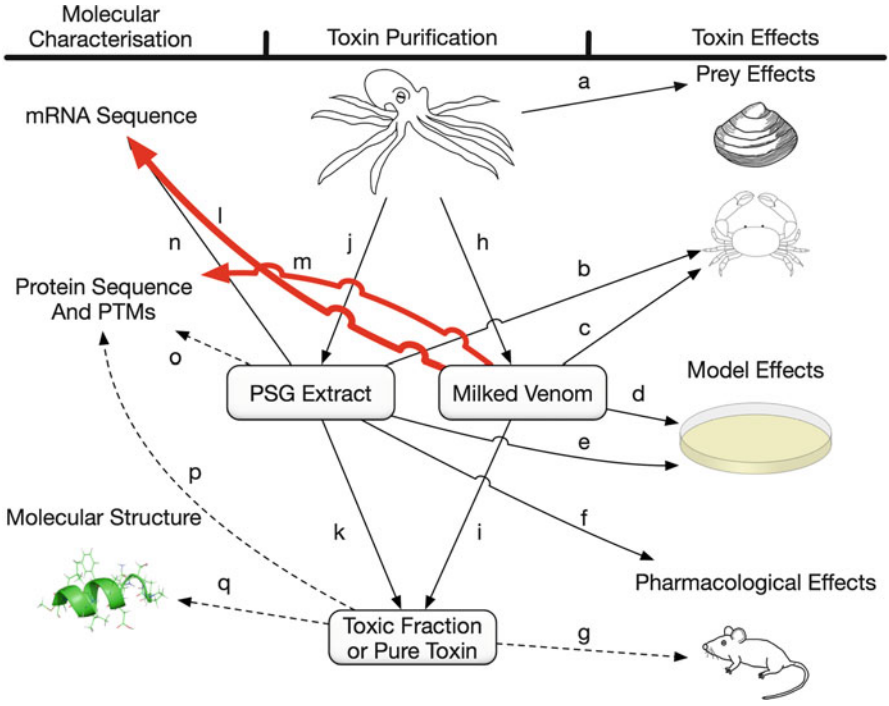

Fig. 3 Summary of research on proteinaceous cephalopod toxins to date. Toxin purification is shown in along the *center line*, with methods for measuring toxin effects shown on the *right*, and for molecular characterization on the *left*. Notable missing lines of inquiry are shown in *thick red* (no studies) or as *dashed lines* (very few studies). Full details describing each labeled *arrow* are given in Table 1 with example studies

An additional issue identified by Fig. 3 is that no venomics studies have been performed on pure cephalopod saliva (as opposed to posterior salivary gland extracts). Obtaining pure saliva is clearly a technical challenge, but one that a number of studies in the past have overcome through milking (Grisley and Boyle 1987) or careful dissection of the venom ducts (Ghiretti 1960). Studies on pure saliva are important because they allow unambiguous identification of venom products without the presence of proteins related to other functions of the posterior salivary gland. Nevertheless, future studies to tackle this issue will need to employ a combined transcriptomic/proteomic strategy since protein production (and hence related mRNA) occurs in the gland and not in the saliva/venom itself. Thus, proteomics of saliva/venom combined with a database of transcript sequences obtained from a variety of body tissues including posterior salivary gland is required.

While venomics studies can identify the protein sequences of many potential toxins simultaneously, their ability to make inferences about the biological significance of these molecules is heavily reliant on homology with sequences where detailed functional studies, or bioassays, have previously been conducted. One of

Table 1 Methodologies and lines of inquiry pursued in cephalopod toxin research. Labels correspond to arrows in Fig. 3

Label	Description	Examples
a	Behavioral and morphological studies demonstrating venom use by cephalopods	Fiorito and Gherardi 1999; Kasugai et al. 2004
b, c	Injection of salivary extract or milked venom into representative prey species (as an assay)	Ghiretti 1960; Songdahl and Shapiro 1974
d, e	Model assays on milked venom or posterior salivary gland extract	Erspamer 1948; Grisley and Boyle 1987; Key et al. 2002
f, g	Investigation of potential medicinal effects of whole venom or a pure toxin isolated from venom	Karthigayan et al. 2007
h	Extraction of pure saliva via milking or dissection	Ballering et al. 1972; Ghiretti 1960
j	Dissection of posterior salivary glands and homogenization and/or extraction with solvent	Erspamer 1948; Cariello and Zanetti 1977
k, i	Isolation of pure active fraction via bioassay-guided fractionation	Anastasi and Erspamer 1962; Cariello and Zanetti 1977; Ghiretti 1959
l, m	mRNA sequencing (transcriptomics) or proteomics (via mass spectrometry) on pure cephalopod saliva	No cephalopod studies. See Corrêa-Netto et al. 2011 for an example in other taxa
n	Transcriptomic sequencing on posterior salivary gland extracts	Fry et al. 2009; Ruder et al. 2013
o	Proteomics on posterior salivary gland extracts	Cornet et al. 2014
p	Direct sequencing of pure toxic peptides or proteins	Anastasi and Erspamer 1963; Ueda et al. 2008
q	Secondary or tertiary structure determination	Grace et al. 2003

the few recent studies to pursue such detailed work is that of Ueda et al. (2008), who used between three and 18 specimens per species to study posterior salivary gland toxins across three cuttlefish and four squid species. This study observed toxic activity in six of seven species and was able to fully isolate and sequence one toxic protein (SE-cephalotoxin) from *Sepia esculenta*. Unfortunately, toxic proteins from squids in this study were not amenable to separation on the basis of salt concentration; however, it is clear that those proteins were potent against both mice and crabs (whereas cuttlefish venom was effective only against crabs) and would be interesting targets for future purification and sequencing efforts.

Conclusion and Future Directions

Research into the nature of toxicity in cephalopods dates back over 100 years with detailed biochemical studies dating from the 1950s. Despite this long history, our understanding of cephalopod venoms, and the toxins they contain, remains extremely rudimentary. In particular, very few examples exist where a particular

molecule has been isolated as a pure (or near pure) fraction, assayed to determine its physiological effects, and then characterized to determine its structure or amino acid sequence. Notable exceptions are the tachykinin eledoisin, SE-cephalotoxin, and TTX. Toxins of octopuses, squids, and cuttlefishes show a very high potential for biodiscovery. The application of "venomics" technologies to toxin research is emerging as an important and powerful way of characterizing entire suites of proteinaceous toxins from pure venom or gland extracts in cephalopods.

Cross-References

- ▶ A Critique of the Toxicoferan Hypothesis
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins
- ▶ Systematics of Cephalopods

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The Strategic Use of Venom by Spiders

8

Allen M. Cooper, David R. Nelsen, and William K. Hayes

Abstract

Understanding the behaviors by which animals deploy their venoms has been largely neglected compared to other aspects of the evolution and biology of venomous organisms and their venoms. Yet, behavior has long been recognized as a pacemaker for the evolution of morphological, ecological, life history, and other traits, in large part because behavioral responses can expose organisms to or protect them from novel selection pressures. The importance of behavior is especially evident in that venom most often functions through a behavioral act that generates a wound in a target animal through which the toxic secretion must be introduced. As a limited and costly commodity, venom should be deployed strategically and judiciously by those animals that possess it. The chapter summarizes the major aspects of adaptive venom use in animals, and highlights the best documented examples of strategic venom deployment among spiders. These animals, like other venomous taxa, exhibit four major behavioral strategies. First, they are often highly selective when using their venom, discharging it only under certain conditions. Second, they can modulate the quantity of venom they expend in both predatory and defensive contexts, delivering multiple bites or variable quantities within individual doses. Third, at least one study suggests that spiders possess venom gland heterogeneity and therefore deliver varying venom composition with successive venom expulsions. Finally, some evidence suggests that

A.M. Cooper (✉) • W.K. Hayes
Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA, USA
e-mail: acooper@llu.edu; whayes@llu.edu

D.R. Nelsen (✉)
Department of Biology and Allied Health, Southern Adventist University, Collegedale, TN, USA
e-mail: dnelsen@southern.edu; dnelsen@llu.edu

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_13

spiders can strategically target the delivery of their weapon at a particularly vulnerable region of their target. Collectively, the evidence suggests a common theme among spiders and other venomous animals for economization and optimization of venom deployment.

Keywords
Chemical ecology • Defense • Predation • Venom economization • Venom metering • Venom optimization

Contents

Introduction

Much of the research on venomous animals and their venoms has focused on the venom delivery systems and the biochemistry, pharmacology, and toxicology of the venoms. Implications for human health, quite naturally, have driven this intense interest and investment. The impetus is evident, for example, among the most studied groups of snakes, for which venomous genera have received far more attention than nonvenomous genera, and the overwhelming majority of publications for the venomous taxa have addressed aspects related to venom rather than other facets of their biology (Beaman and Hayes 2008).

Researchers have also investigated the behaviors associated with venom deployment, but this important aspect of the evolution and biology of venomous animals remains relatively neglected. The notion that behavior contributes substantially to the evolutionary process dates to Lamarck (1809) and has been developed further by subsequent Darwinian expositors (reviewed by Corning 2014). Behavior has been viewed by many as the "pacemaker of evolution" because behavioral responses can expose organisms to or protect them from novel selection pressures, thereby influencing the evolution of morphological, physiological, life history, and other traits (Duckworth 2009; Wcislo 1989; Wolf and Weissing 2012). Indeed, the individual's environment is influenced substantially, for example, by its movements, choice of habitat, feeding behavior, predator avoidance, mating strategy, and social behavior. Moreover, the behavior of individuals affects population-level properties such as spatial distribution, social structure, trophic interactions, community structure, and ecosystem function. The importance of behavior to the evolution of

venomous animals is especially significant because venom most often functions through a behavioral act that generates a wound in a target animal through which the toxic secretion must be introduced (Nelsen et al. 2014b). Clearly, the study of behavioral phenotypes, mechanisms, and adaptations of venomous animals is critical to understanding the factors that shape the evolution of venom and its associated non-behavioral traits.

Venoms comprise toxic secretions that organisms deliver directly to the tissues of other organisms via physical contact and generation of a wound (Nelsen et al. 2014b). Venom can be distinguished from other toxic secretions that are administered passively by contact or ingestion (poisons) or are transferred by a delivery mechanism to a target's surface without creation of a wound (toxungens). These distinctions underscore the importance of behavior in toxin delivery, but a given secretion may nonetheless function in two or all three of these modes (Nelsen et al. 2014b). Venoms, which may be comprised of a single toxin or a mixture of different toxins, and can be synthesized autogenously or acquired exogenously, interact with the target organism's internal milieu to bring about rapid pathophysiological changes. Animals employ venom for a variety of purposes, including predation, defense, competition for space and mates, and, secondarily for some animal groups, hygiene, communication, and potentially other roles (Mebs 2002; Nelsen et al. 2014b).

Because venom is both a valuable resource and a limited commodity, selection should favor the behavioral use of venom in ways that maximize effectiveness and minimize waste (Hayes 2008; Hayes et al. 2002; Hostettler and Nentwig 2006; Morgenstern and King 2013). Cost-benefit analyses are essential for understanding the adaptive value of behavior because natural selection favors strategies that have a propitious cost-benefit ratio (Cuthill and Houston 1997). The benefits of venom are obvious, particularly for predation and defense (e.g., Mebs 2002; Nelsen et al. 2014b). However, the synthesis of venom, its storage, and its compartmentalization to avoid autotoxicity all entail costs that have only recently been analyzed (reviewed by Morgenstern and King 2013). Regeneration of spent venom may also require a few days or weeks, during which time the animal may be disadvantaged (Morgenstern and King 2013). Beyond the metabolic cost of generating venom, there are ecological costs to venom use as well. These include unnecessary or excessive discharge that can temporarily impair an organism's ability to defend itself or take advantage of future opportunities to procure prey (Hayes 2008; Malli et al. 1998). Venom use is also associated with serious risk of bodily injury, and possibly death, as envenomation requires direct physical contact that can lead to retaliation (Schmidt 1990).

For an organism to deploy venom in an optimal or strategic manner, it must accurately assess both its external environment, including the intended recipient of the venom, and its own internal state (Hostettler and Nentwig 2006; Wullschleger and Nentwig 2002). External factors assessed for predatory venom deployment may include type of prey (Hayes 1992; Wigger et al. 2002), prey size (Edmunds and Sibly 2010; McCormick and Polis 1990; Steiner 1986), and prey struggle intensity or duration (Djieto-Lordon et al. 2001; Malli et al. 1999; Steiner 1986). External factors

assessed for defensive venom deployment may include type of predator or aggressor (Carlin and David 1989), degree of threat (Haight 2006; Nisani and Hayes 2011), and persistence of threat (Fink 1984). Internal factors that may influence venom deployment include the amount of venom remaining in storage (Wullschleger and Nentwig 2002) and satiety level (Hayes 1993). In many cases, the venomous animal may physically interact with the intended venom recipient prior to venom deployment, thus enabling assessment (e.g., struggling prey receive more bites or stings; Malli et al. 1999; Steiner 1986). However, in other cases, such as with snakes that inject venom during a single brief bite, assessment of the target and decisions regarding deployment may be made prior to contact with the receiver (Hayes et al. 2002).

The act of venom deployment, whether it occurs via a simple stimulus-response reflex or by more complex decision making (Jackson and Cross 2011), potentially including cognition, which remains an ill-defined concept (Menzel 2013; Perry et al. 2013; Shettleworth 2013), is generally triggered by stimuli that exceed a threshold. In the case of predation, subthreshold stimuli arising from relatively small, harmless, or unresponsive prey may result in prey capture and consumption without envenomation (Hayes et al. 2002; Malli et al. 1998; Wigger et al. 2002). For defense, subthreshold stimuli may evoke alternate presumably less costly behaviors, including fleeing (Gibbons and Dorcas 2002; Nelsen et al. 2014a), leg autotomy (Nelsen et al. 2014a), threat displays (Gibbons and Dorcas 2002), retaliatory pinching/biting (Heatwole 1967; Schmidt 1990), sham strikes (Hayes 2008), and dry bites or stings (Herzig 2010; Morgenstern and King 2013; Nisani and Hayes 2011).

Venomous animals can strategically deploy their venom in four ways. First, presumably all venomous animals are highly selective when using their venom, discharging it only under certain conditions. In some cases, behavioral regulation of venom release is sufficient that the full act of venom delivery may ensue without actual release of venom, resulting in a dry bite or sting. Second, animals can modulate the amount of venom they expend, delivering more in some circumstances and targets and less in others. This can be accomplished in either of two ways: by varying the number of bites or stings or by varying the quantity of venom expended with each bite or sting. The capacity of animals to modulate venom quantity has been described as the "venom metering" (Hayes 2008; Hayes et al. 2002) or "venom optimization" (Wigger et al. 2002) hypotheses. Third, at least some venomous animals can alter the composition of venom depending on context of use, as occurs most notably in cone snails (Dutertre et al. 2014). Scorpions also possess venom heterogeneity, but venom composition during successive stings covaries with quantity of venom (Nisani and Hayes 2011). Finally, venomous animals may strategically target their venom by aiming it in the direction of an intended victim, or even delivering it to a particularly vulnerable body part (Libersat and Gal 2014; Malli et al. 1998, 1999). This targeting can potentially reduce the quantity of venom used, particularly for predation.

The purpose of this review is to summarize the major aspects of adaptive venom use in spiders and highlight the best documented examples of strategic venom deployment among this diverse group. Spiders (class Arachnida, order Araneae) comprise one of the most studied groups of venomous animals and serve as excellent

Table 1 Documented examples of strategic venom use by spiders. Parenthetic examples are explained in footnote. See text for references

Strategic venom deployment	Predation	Defense
Selective venom use	✓	✓
Number of doses	✓	✓
Volume per dose	✓	✓
Venom composition	(✓)	(✓)
Delivery location	✓	

Spider venom composition, like that of scorpions, appears to be heterogeneous (Morgenstern et al. 2012); thus, their venom composition presumably covaries with venom quantity and therefore is indirectly modulated

models for exploring the behavioral use of venom. Almost all spiders are venomous, with only a few families having lost their venom glands. Many species, particularly those among the araneomorphs, have invested heavily in the use of venom. Venom has allowed spiders to become very successful predators. They are often among the top terrestrial predators of arthropods, but some species are capable of capturing much larger vertebrate prey. Sufficient evidence has now accumulated to provide compelling evidence that, despite their relative neurological simplicity (compared to venomous vertebrates), spiders can utilize each of the four aforementioned aspects of strategic venom delivery, as summarized in Table 1.

Spider Venom Apparatus and Venom

Spiders possess a pair of fangs attached to the chelicerae that they use to deliver venom via biting. The venom gland may be exclusively located within the chelicerae (as in the mygalomorphs), but may also extend well into the cephalothorax, occupying a significant proportion of the total volume (as in the araneomorphs; Nentwig and Kuhn-Nentwig 2013). Cheliceral fangs act as hypodermic needles, typically opening at their distal tip and usually moving in one of two planes: either dorsoventrally in the mygalomorphs (infraorder Mygalomorphae, or Orthognatha) or mediolaterally in the araneomorphs (infraorder Araneomorphae, Labidognatha). Spider venoms comprise complex, multi-component mixtures of biologically active substances, including neurotransmitters and salts, acylpolyamines, peptides, and proteins (including some of large molecular weights; Casewell et al. 2013; Fry et al. 2009; Kuhn-Nentwig et al. 2011), that play important roles in both predation (killing or paralyzing prey) and defense (Foelix 1996; Nentwig and Kuhn-Nentwig 2013; Quintero-Hernandez et al. 2011).

Cost of Venom in Spiders

Despite the value of venom to spiders, no quantitative data exist on the metabolic cost of its regeneration, although Malli et al. (1999) suggested venom production is energetically expensive. Nevertheless, data on other aspects of venom regeneration

suggest that spiders incur an ecological cost for venom expenditure. Because venom regeneration may take weeks (Boeve et al. 1995; Perret 1977b) to months (Freyvogel et al. 1968), and spiders may capture several prey items per day, spiders should modulate venom release to avoid the metabolic expense of regenerating depleted reserves, which could leave the spider vulnerable to predators or unable to deal with subsequent prey (Boeve and Meier 1994; Malli et al. 1998). The secondary loss of venom in uloborid spiders, which kill their prey instead by wrapping them tightly in hackled silk, further suggests that venom use comes with a considerable biochemical price (Morgenstern and King 2013).

Selective Venom Use by Spiders

Strategic deployment of venom by spiders includes using this valuable commodity selectively. Spiders appear to be capable of choosing whether to use any venom at all. Withholding venom can occur in two contexts: by crushing prey with the chelicerae without employing the fangs, or by using the fangs without concomitant venom expulsion. The ability to use or withhold venom independent from fang use stems from spider anatomy. The venom glands are surrounded by striated muscle under nervous control, allowing the deployment of venom via muscular contraction of the gland at the volition of the spider (Boeve et al. 1995; Bucherl 1971; Malli et al. 2000; Schenberg and Pereira-Lima 1978).

In the context of predation, the interplay between prey size, prey defensive capabilities, and capacity of prey to struggle will influence whether spiders deploy their venom. In a number of species investigated, prey size represents an important factor influencing venom deployment, with spiders routinely seizing and chewing small arthropods without applying venom, relying instead on the chelicerae to crush or chew them, thereby reserving venom for larger prey (Cupiennius salei [Ctenidae]: Malli et al. 1998; Nentwig and Kuhn-Nentwig 2013; Wigger et al. 2002; Argiope argentata [Araneidae]: Robinson 1969; Phoneutria nigriventer [Ctenidae]: Schenberg and Pereira-Lima 1978). Malli et al. (1998) quantified the venom dose injected by *C. salei* (Ctenidae) into crickets of various size classes ranging from 100 to 660 mg. They found that the spiders did not inject venom into 22 % (7/32) of the crickets bitten in the smallest size class (100–110 mg). The authors contended that *C. salei* does not rely exclusively on its venom when feeding on small prey, but can accomplish the job through mechanical damage alone inflicted by the chelicerae. Prey size is also important for *P. nigriventer* (Ctenidae), which only injects venom into excessively large prey, relying on mechanical damage caused by the chelicerae to kill small insects (Schenberg and Pereira-Lima 1978). According to Wigger et al. (2002), the difficulty a spider encounters in overwhelming prey, which can vary with prey species, may also determine whether spiders use their venom. Wigger et al. (2002) documented selective venom use by *C. salei* while using an enzyme-linked immunosorbent assay (ELISA) to quantify the amount of venom the spider injected into four different prey (blowflies, crickets, stick insects, and ground beetles). The authors found that no venom could be detected in 32 % (6/19) of the crickets

attacked, whereas all individuals of the other prey types that were attacked had been envenomated. The authors suggested that sometimes the spider relies on its strong chelicerae to kill soft prey susceptible to mechanical damage. However, it remains unclear why, if *C. salei* often withheld venom from crickets, it did not also occasionally withhold venom from stick insects, which the authors argued were also a soft, unproblematic prey type. Other investigators have also noted the influence of prey struggle (Bücherl 1971) and the prey's defensive capabilities (Kuhn-Nentwig et al. 2011) on whether venom is used.

Spiders may selectively deploy venom in defense as well. For example, *P. nigriventer* employs venom only when the spider finds no way to escape attack (Schenberg and Pereira-Lima 1978). In female mouse spiders, *Missulena bradleyi* (Actinopodidae), aggravation by experimenters led to voluntary expression of venom from only 15 % of spiders, suggesting subthreshold stimulation for venom expenditure in most cases (Herzig et al. 2008). Defensive dry bites represent another example of selective use of venom. Herzig (2010) argued that some dry bites by the mouse spider (*Missulena* spp.) investigated by Isbister (2004) could be explained by the voluntary decision of the spider not to deploy venom during a bite in order to save the metabolic expense of venom synthesis. While analyzing methods of venom extraction from the African tarantula *Scodra griseipes* (Theraphosidae), Celerier et al. (1993) observed that the spiders could bite a lure many times without emitting venom. Freyvogel et al. (1968) similarly noted that the baboon spider *Pterinochilus* sp. (Theraphosidae) often actively withheld venom during milking attempts. Nelsen et al. (2014a) investigated defensive venom use in the western black widow spider (*Latrodectus hesperus*) and found that, when pinched, at least 50 % of the bites to three successive presentations of parafilm-covered tubes appeared to be dry. The proportion of dry bites did not decline among the three targets in succession, and dry bites often preceded wet bites (Table 2), suggesting that the spiders deliberately

Table 2 Selective venom use. Sequence of venom usage (dry vs. wet bites) by western widow spiders (*Latrodectus hesperus*) when defensively biting three targets in succession ($N = 80$ trials) separated by brief (5 s) or lengthy (5 min) intervals (Adapted from Nelsen et al. 2014a)

			Frequency		
Target 1	Target 2	Target 3	Brief intervals	Lengthy intervals	Plausible interpretation
Dry	Dry	Dry	15	7	
Dry	Dry	Wet	8	7	Venom metering
Dry	Wet	Dry	7	6	Venom depletion
Dry	Wet	Wet	1	9	Venom metering
Wet	Dry	Dry	5	3	Venom depletion
Wet	Dry	Wet	3	4	Venom metering
Wet	Wet	Dry	1	1	Venom depletion
Wet	Wet	Wet	0	3	

Venom metering: dry bites preceded wet bites, indicating available venom and decision making
Venom depletion: dry bites resulted from prior venom use that depleted reserves

withheld their venom. Taken together, these data indicate that decisions about venom deployment depend on several factors, including prey size, prey type, and threat level.

Amount of Venom Deployed by Spiders

Spiders have been compared to snakes in their ability to control the amount of venom delivered (Schenberg et al. 1970). Evidence suggests that the degree of venom gland emptying is at the spider's volition (Boeve et al. 1995; Maretic 1987). Using indirect measures of the amount of venom deployed, investigators have found that prey size and struggle intensity are important factors influencing predatory venom expenditure. For example, Perret (1977a), comparing volume of venom electrically milked before and after spiders were fed, found that the tarantulas *Aphonopelma chalcodes* and *Dugesiella hentzi* released more venom in the first bite when feeding on adult (1–2 g) cockroaches (*Periplaneta americana*) than when feeding on adult (0.1 g) mealworm beetles (*Tenebrio molitor*). When attacking cockroaches, *A. chalcodes* injected, on average, 1.7 μL of venom (25 % of available venom), but injected no venom into mealworm beetles. Similarly, *D. hentzi* injected 1.7 μL of venom (28 % of available venom) into cockroaches, but only 0.1 μL of venom (2 % of available venom) into mealworm beetles. As the venom was delivered in a single bite, the mechanism of venom deployment is likely related to the extent of venom gland compression or the number of compressions. In another study, based on mass gain of bitten prey, Pollard (1990) found that the New Zealand crab spider *Diaea* sp. (Thomisidae) injected more venom into a larger fly, *Pegohylemyia* sp., than into the much smaller fruit fly *Drosophila immigrans* (0.108 vs. 0.067 mg venom, respectively). In only 7 % (3/43) of cases was a prey item bitten more than once, indicating that number of bites was not the primary mechanism for controlling amount of venom deployed. Although the author noted that *Diaea* can regulate the amount of venom injected based on prey size, he also suggested that the spider may use tactile information from captured, struggling prey to help assess prey size.

Boeve (1994) investigated the mortality rates of multiple series of crickets (*Acheta domesticus*) of different mass classes attacked by *C. salei*. He found that for prey of small or medium mass (less than 40 mg), the spider injected an amount of venom proportional to the mass of the prey, whereas large prey items were injected with all its venom. Furthermore, by interrupting bites on different prey size classes at various intervals and analyzing the state of bitten prey, the author showed that the spiders varied the rate of venom delivery in a single bite based on prey mass. The author further speculated that each escape attempt may have stimulated the spider to inject a discrete amount of additional venom. In another study using *C. salei*, and based on similar methods, Boeve et al. (1995) demonstrated that larger crickets received larger venom doses than smaller crickets. Furthermore, the authors showed that *C. salei* injected larger venom doses into "difficult-to-handle" prey (cricket *Gryllodes sigillatus*) than into "easy" prey (cricket *Gryllus bimaculatus*), with the dichotomy essentially reflecting a difference (not quantified) in struggle intensity

after attack. The authors concluded that *C. salei* could empty its glands partially or completely, resulting in dosed or metered injections of venom. Thus, several studies using indirect measurements of venom expenditure suggest that the amount of venom deployed by spiders varies with prey size and struggle intensity.

Malli et al. (1998) were the first to directly quantify spider venom expenditure into various size classes of prey. The authors performed ELISA on whole-cricket (*A. domesticus*) homogenates using monoclonal antibodies to the main toxin in *C. salei* venom, CSTX-1. Their results (Fig. 1a) revealed that, when mature *C. salei* females attacked crickets ($N = 128$ attacks by 16 spiders) of four size classes (12, 15, 18, 22 mm), a significant relationship existed between the size of prey and the quantity of venom expended ($r = 0.80$), with mean venom quantities ranging from 0.15 μL for the smallest prey to 1.53 μL for the largest. Multiple comparisons indicated that *C. salei* released significantly more venom with increasing size of cricket ($p < 0.01$ for all comparisons). Although a clear relationship between venom dose and prey size was found, the authors acknowledged it remained unclear whether more venom was injected into larger prey simply because of size, or as a consequence of greater struggle by larger prey. The authors also suggested that the pattern of venom deployment could result from some combination of *C. salei* injecting venom gradually until prey is motionless and a size-based difference in venom susceptibility. The authors did not emphasize number of bites delivered to prey, although multiple bites did occur in at least 5 % of attacks. Thus, a metering mechanism based on a gradually delivered dose of venom from a single bite was more common than one based on multiple bites. In a follow-up study, Malli et al. (1999) further investigated the influence of prey size on venom expenditure by mature female *C. salei*, once again using an ELISA. To disentangle the effects of prey size and struggle intensity on venom dosage, the authors used anesthetized crickets (*A. domesticus*) in four size classes (100–110, 290–320, 420–460, and 600–660 mg) that were artificially induced to struggle at the same intensity for a set duration (5 min). Quantity of venom released varied widely within a size class, and prey size and quantity of venom expended were weakly correlated ($r = 0.23$, $p < 0.05$). Multiple comparisons revealed that *C. salei* injected significantly less venom into the smallest size class, whereas no significant differences were found among the other size classes. The authors concluded that prey size alone is not likely to be an important cue for regulating venom injection in this spider. Further, they argued that the results of Malli et al. (1998), in which larger prey received larger venom doses, were a consequence of predator-prey interactions during envenomation which, though increasing with size of prey, did not depend on the size of the prey itself.

In addition to prey size, Malli et al. (1999) investigated the effects of prey struggle intensity and prey struggle duration on the amount of venom *C. salei* injected. Using ELISA to quantify the venom injected into anesthetized crickets of the same mass (290–320 mg) that were experimentally manipulated to struggle at four different intensities (no movement [control], low, medium, and high), the authors found a highly significant relationship between intensity of prey movement and quantity of venom expended. Multiple comparisons indicated that, with the exception of the

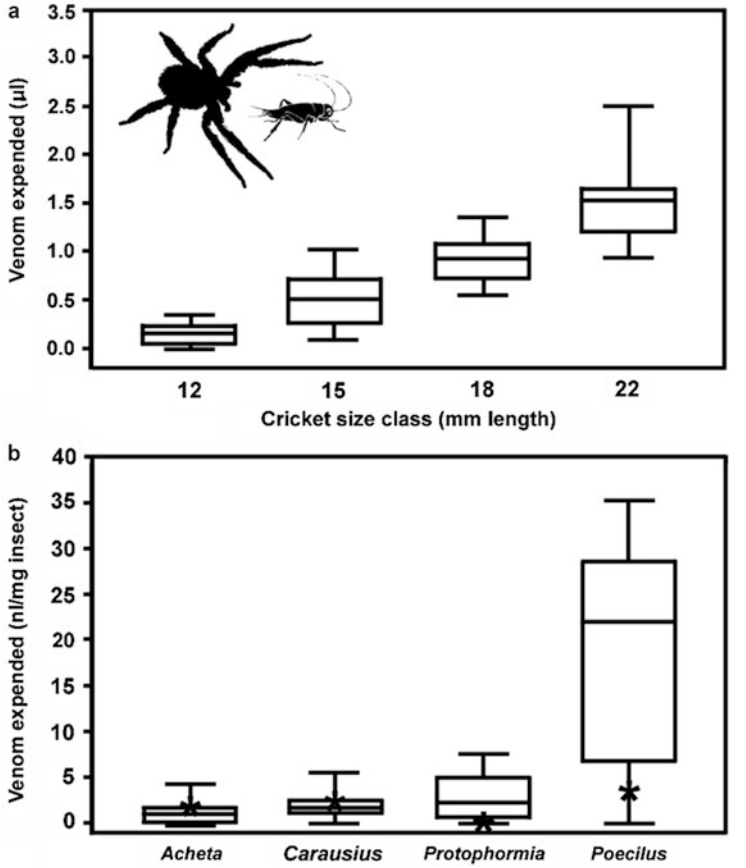

Fig. 1 Modulation of venom quantity based on prey size and species. (**a**) Volume of venom injected by the spider *Cupiennius salei* ($N = 16$) into four size classes of crickets, as determined by enzyme-linked immunoassay (ELISA) of whole-cricket homogenates. *Box* plots show the first quartile, median, and third quartile. *Error bars* represent the 10th and 90th percentiles. Note the correspondence between prey size and venom expended (Adapted from Malli et al. (1998)). (**b**) Volume of venom injected by *C. salei* into four different prey genera, adjusted for prey body mass. *Box* plots are as above, except that *error bars* indicate minimum and maximum values. *Asterisks* represent median lethal dose (LD_{50}) of venom for each prey type. Spiders expended venom doses similar to the LD_{50} value when feeding on larval crickets (*Acheta domesticus*) and stick insects (*Carausius morosus*), but delivered greater doses of venom relative to LD_{50} values for blowflies (*Protophormia* sp.) and ground beetles (*Poecilus cupreus*), which were more difficult to subdue (Adapted from Wigger et al. (2002))

difference between the control and the low-intensity prey movement condition, *C. salei* released significantly more venom as the intensity of prey movement increased. The authors suggested that injection of larger quantities of venom into vigorously resisting prey would induce rapid immobilization, thus preventing

injuries to the spider and/or lost prey. Additionally, the authors noted that *C. salei* saved up to 50 % of its venom by discriminating between high- and low-intensity prey movements.

Malli et al.'s (1999) study of the influence of prey struggle duration on venom expenditure by *C. salei* yielded similar results. In this experiment, the crickets (290–320 mg) were vibrated at the same intensity (medium) but for different lengths of time (0 [control], 1, 2.5, or 5 min) following the initial bite. Data indicated that the duration of prey movement and quantity of venom expended were positively correlated ($r = 0.61, p < 0.01$). Multiple comparisons showed that, with the exception of the difference between the control and 1-min treatments, *C. salei* released significantly more venom with increasing duration of prey movement. Malli et al. (1999) concluded that *C. salei* injects venom gradually in response to stimuli generated during the course of envenomation. The authors speculated that perhaps tactile hairs and slit sense organs found on the chelicerae and base of the claws serve a vibrosensitive function in controlling the release of venom during envenomation. For all experiments, the mechanism for varying venom expenditure was independent of number of bites, because the prey were held in the chelicerae (i.e., single bite) for the duration of each trial.

Following in the footsteps of Malli et al. (1999), Wigger et al. (2002) demonstrated differential venom expulsion by *C. salei* based on prey species. The authors used ELISA to quantify venom injected by adult female *C. salei* into four prey species: blowflies (*Protophormia* sp.), larval crickets (*A. domesticus*), stick insects (*Carausius morosus*), and ground beetles (*Poecilus cupreus*). All prey were of a uniform (but unreported) size class. Their results (Fig. 1b) indicated that ground beetles received significantly more venom than the other three prey species. The authors argued that the blowflies, crickets, and stick insects were relatively soft and thus unproblematic prey types, resulting in a relatively low dose of venom. In contrast, the heavily sclerotized ground beetles represented difficult-to-overwhelm prey because spiders were forced by the beetles' mechanical protection to inject their neurotoxic venom into the prey's abdomen, an injection site requiring more venom to subdue the prey than a bite to the head or thorax normally would. In fact, the authors suggested that the lengthy handling time for ground beetles may have been the stimulus leading to greater venom expenditure. Although the number of bites *C. salei* delivered to prey was not stated, spiders appeared to hold prey in their chelicerae (i.e., single bite) for each 5-min trial, indicating that the mechanism controlling venom expenditure was independent of number of bites.

Risk of prey escape, which may vary with prey species, may also influence venom deployment. Robinson's (1969) findings suggest this possibility, and Boeve et al. (1995) interpreted them in this way, citing the study as evidence that more venom is injected into easily escaping insects. Robinson (1969), while studying the predatory behavior of *A. argentata*, noted that lepidopteran prey, which were bitten prior to silk wrapping, received a statistically longer bite than other prey types, which were first wrapped in silk and then bitten. It was suggested that the short bite might deliver a smaller dose of venom (Robinson 1969; Robinson et al. 1969;

Robinson and Olazarri 1971) because it would be wasteful to use biologically expensive secretions unnecessarily on a wrapped prey item (Robinson 1969). However, the long bite may be long simply because the spider must wait for the venom to take effect before it can safely release the prey and commence wrapping (Robinson et al. 1969). The adaptive significance of the long bite lies in its ability to cause the most rapid restraint of prey with high escape potential, such as lepidopterans (Robinson 1969; Robinson and Olazarri 1971). Although the duration of the long bite delivered to lepidopterans varied dramatically (e.g., from 1 to 527 s when attacking live moths; Robinson and Olazarri 1971), there was no systematic relationship between length of bite and weight of prey for either the long or short bite (Robinson 1969).

Wullschleger and Nentwig (2002) experimentally examined whether adult females of *C. salei* "know" how much venom is available in their venom glands and make predatory decisions accordingly (Fig. 2). They emptied the venom glands of their contents by either electrical milking or by allowing the spider to bite three crickets and compared these spiders to control animals having replete (full) venom glands. When presented with two prey items simultaneously, adult female *C. salei* spiders shifted their attacks toward the cockroach species that was more easily

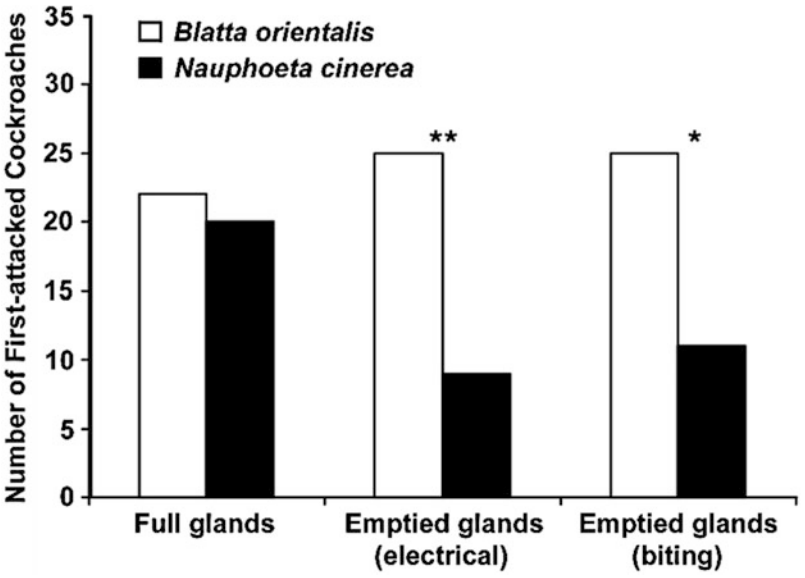

Fig. 2 Modulation of predatory attack based on venom supply. When presented with two prey items simultaneously, adult female *Cupiennius salei* spiders shifted their attacks toward the cockroach species more easily subdued by venom (*Blatta orientalis*, *white bars*) and avoided the more venom-resistant species (*Nauphoeta cinerea*, *black bars*), after experimental depletion of their venom. Venom glands were emptied by either electrical milking or by allowing the spider to bite three crickets. *$p < 0.05$, **$p < 0.01$ (chi-square tests) (Adapted from Wullschleger and Nentwig (2002))

subdued by venom (*Blatta orientalis*) and avoided the more venom-resistant species (*Nauphoeta cinerea*). Hostettler and Nentwig (2006) subsequently showed that *C. salei* uses olfactory information to identify prey type and distinguish venom sensitivity, presumably to conserve venom.

Although much of the work examining venom deployment in spiders has focused on the circumstances of a single sustained bite, spiders do sometimes bite prey multiple times (Gilbert and Rayor 1985; Malli et al. 1998; Parks et al. 2006; Schenberg and Pereira-Lima 1978; Willey et al. 1992). Thus, varying the number of bites represents an additional means by which spiders could control the amount of venom deployed. In such cases, continued prey struggle may be the stimulus for additional bites (Gilbert and Rayor 1985).

In the context of defense, venom metering is less well studied. Fink (1984) found that the green lynx spider *Peucetia viridans* (Oxyopidae) varies the amount of venom expended based on extent of provocation and may vary the amount of venom in individual spits. Female spiders ejected venom forward up to 20 cm when approached or when their legs were pulled. Although a single spit was most common, spiders would spit several times in succession if repeatedly provoked. The quantity of venom in a spit was variable, from trace amounts to more than 5 μL. In tarantulas, Perret (1977a) investigated the amount of venom released in a single bite. In defensive bites against mice (30 g; $n = 2$ cases), *A. chalcodes* injected, on average, 2.3 μL of venom (36 % of available), about the same amount of venom (1.7 μL, 25 % of available) that they injected, when feeding, into cockroaches, but more than they injected into mealworm beetles. The author suggested that since cockroaches and mice were of considerably different sizes, and spiders displayed typical defensive behavior toward mice, it was possible that the spider calculated venom injection differently in defensive compared to predatory situations.

More recently, Nelsen et al. (2014a) used ELISA to quantify the venom delivered by *L. hesperus* during defensive encounters, investigating how presumed level of threat (body vs. leg pinch) and persistence of threat (brief vs. lengthy intervals between three successive simulated attacks) affected venom expenditure. The spiders injected 1.8-fold more venom per bite when pinched on the body compared to a leg (Fig. 3). Body pinches presumably would be perceived as a higher threat than leg pinches, because the body contains the vital organs and leg autotomy would be an alternative strategy to venom use. The spiders also deployed 2.3-fold more venom when the three successive threats were separated by brief (5 s) compared to lengthy (5 min) intervals. The authors suggested that the spiders treated the brief intervals as a single predatory encounter and the lengthy intervals as separate events requiring additional venom for each new attacker. Nelsen et al. (2014a) concluded that their results were consistent with risk assessment and the capacity to modulate venom expenditure during defensive encounters.

Considering this large body of evidence, there can be little doubt that some spiders have the capacity to vary the amount of venom expended during predatory contexts. Perhaps unsurprisingly, the responses of bitten prey comprise an important cue influencing how much venom spiders inject. The evidence for venom metering

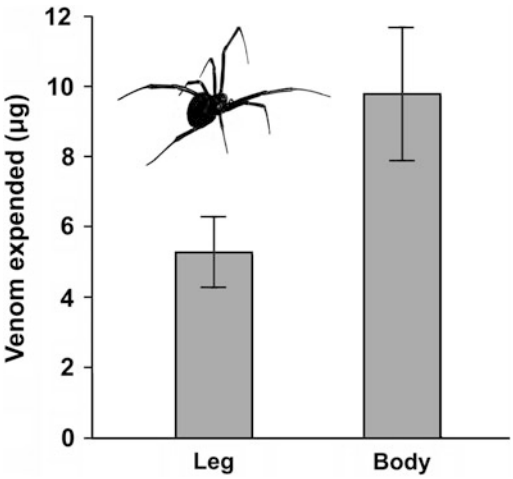

Fig. 3 Modulation of venom quantity based on presumed threat assessment. Mean (±1 S.E.) mass of venom expended during defensive bites by adult females of the western black widow spider (*Latrodectus hesperus*) when pinched on different body parts by investigators. Spiders delivered 1.8-fold more venom when the more vulnerable body was pinched compared to when an expendable leg was pinched (Data from Nelsen et al. (2014a))

within a defensive context is weaker, with fewer studies having examined the possibility. Nevertheless, spiders appear capable of risk assessment and making decisions about the quantity of venom to use.

Modulation of Venom Composition in Spiders

Recent research hints that certain (and possibly all) spider species, similar to scorpions (Nisani and Hayes 2011), are capable of expulsing venom of heterogeneous composition. To date, a single study has found that venom of the funnel web spider (*Hadronyche infensa*) ejected early in a bite sequence differs significantly in protein composition from venom delivered later in a bite sequence (Morgenstern et al. 2012). If venom expulsion heterogeneity is widespread in spiders, then venom composition may simply covary with quantity of venom expended and therefore be modulated only indirectly, as occurs in scorpions (Nisani and Hayes 2011). Venom expulsion in this fashion results presumably from a relatively large, fairly stationary, slowly replenished venom pool that is differentially synthesized and stored regionally along the length of the venom gland. However, further study may reveal rapid and reversible venom composition changes associated with venom use, which has been documented in cone snails (Dutertre et al. 2014). In the snails, differences in stimulation between predatory and defensive contexts apparently promote rapid venom secretion from different regions of the gland into a relatively small lumen, which is then flushed with each venom expulsion event. The different regions of the gland synthesize unique venom components, resulting in substantially different venom composition among successive venom expulsions. One spider group, the spitting spiders (*Scytodes*, Scytodidae), also possesses regional heterogeneity of their venom gland, with the anterior portion producing adhesive, silken strands apparently devoid of toxicity that are spat to immobilize prey, and the posterior

portion producing a toxic venom that is delivered by injection to further incapacitate or kill their prey (Zobel-Thropp et al. 2014). Both secretions are extruded through the same duct. Because the glue-like material must pass through the anterior portion of the gland without picking up toxicity, this suggests a mechanism similar to that of cone snails, and the widespread possibility among spiders of rapid and reversible venom composition changes with successive bites.

Strategic Targeting of Venom by Spiders

Delivery location of venom may be an important part of strategic venom deployment in some spiders. However, without data on prey morphometrics, it can be difficult to distinguish spider targeting preference from an unequal, but still random, bite distribution stemming from unequal surface areas of body parts available for biting (Morse 1999). Furthermore, for spiders, evidence suggests that initially attempted bite location may often play a smaller role in prey incapacitation than the final location of envenomation (Malli et al. 1998; Pollard 1990). One might suspect that a strategic site of venom injection used by spiders, given the potent neurotoxins present in their venoms (King and Hardy 2013), would be near the prey's central nervous system, typically the thorax or head. In general, thorax envenomations are common (Foelix 1996), and several investigators have contended that targeting the thorax or head produces the fastest effects (Malli et al. 1998, 1999; Pollard 1990; Wigger et al. 2002). In fact, as Morse (1999) pointed out, such a "neckbite" pattern of envenomation is often reported by general spider sources. Even so, data on prey bite location remain relatively scarce.

Much of the limited data on location of predatory spider bites comes from the spider *C. salei*. Malli et al. (1999) reported that when *C. salei* was offered CO_2-anesthetized crickets (*A. domesticus*), the majority of the prey were bitten in the thoracic region (87.4 % [420/480] of bites), whereas only 12 % and 0.6 % of bites were delivered to the abdomen and head, respectively. In a separate study, Malli et al. (1998) reported the frequency of bites to a given body region varied among prey size classes, but most crickets were bitten either in the thorax (66–85 %) or the pronotum right behind the head (9–28 %). In contrast, few bites occurred on the abdomen (3–9 %) or the chitinized head capsule (0–3 %). Furthermore, except in one case, all crickets first bitten in the abdomen were subsequently bitten also in the thorax. Given that a cricket's thorax is smaller than its abdomen, the reported distribution of bites suggests a preference for thorax envenomation. Malli and colleagues (Malli et al. 1998, 1999) argued that bites to the thorax may decrease the amount of venom needed for paralyzation and reduce time to immobilization, thereby reducing the spider's risk of injury. In contrast to the high frequency of thorax bites to crickets, when *C. salei* preyed on heavily sclerotized ground beetles (*P. cupreus*), the spiders, after attempts to deliver a bite to the thorax or head, most often ended up biting the abdomen (Wigger et al. 2002). This study demonstrates that strategic deployment of venom by delivery location can be constrained by prey characteristics.

Other spiders besides *C. salei* appear capable of targeting their venom. Pollard (1990) noted that more than half a dozen species of crab spiders are known to envenomate prey principally in the head and thorax, and several species have been observed to re-envenomate prey in the head or thorax following initial capture by envenomation of the abdomen. This author also hypothesized that crab spiders re-envenomate prey in the thorax to achieve faster immobilization. The orb-web spider *Argiope aurantia* may likewise strategically envenomate certain prey (Harwood 1974). After wrapping orthopteran prey, this spider delivers a series of short bites and a single sustained bite. Data for 51 bites indicated the majority (~80 %) of sustained bites were on the anterior half of the prey. For the orb-web spider *A. argentata*, non-lepidopteran prey were bitten after wrapping, and bites were often directed at the head or thorax (Robinson and Olazarri 1971). However, in some cases initial bite location is more a matter of happenstance; when prey were bitten before wrapping, which is common for lepidopteran prey, the bite was often delivered to the first point of prey contact. Even so, if the initial bite occurred on a wing or other appendage, the bite was transferred to a more "substantial" part of the prey, possibly due to sensory information received directly by the chelicerae.

In contrast to the above examples in which spiders targeted their venom toward the prey's central nervous system, the bites of other spiders may be directed toward peripheral targets such as legs or antennae (Parks et al. 2006; Suter and Stratton 2012), or directed seemingly randomly toward prey in the same proportion as the surface area of the prey's body parts (Morse 1999). Bites directed toward the legs appear to be particularly efficacious for the ant-eating spider *Zodarion cyrenaicum* (Zodariidae). Both the young and adults of this species subdued exceptionally large prey, more than 30 times their own mass, with one or two highly toxic bites that were delivered 75 % of the time to a leg (Pekar et al. 2014). Most of the bites to legs (60 %) were delivered to a rear leg, which presumably afforded greater safety.

Taken together, sufficient evidence indicates that many spiders demonstrate a preference for envenomating certain types of prey in a particularly vulnerable region, presumably to effect rapid prey immobilization, or in a relatively safe region to avoid retaliatory injury. However, targeted venom delivery in spiders is neither strict nor universal.

Conclusions and Future Directions

The widespread reliance on venom for predation and defense among spiders underscores the important role of this toxic secretion in the biology of this group. Two common themes emerge from this review of venom deployment: economization and optimization of venom. Accumulating evidence from spiders supports the view that venom represents a valuable but limited commodity. Although spiders are ideally suited to measure the metabolic costs associated with synthesis, storage, and discharge of their venom, no empirical study has been conducted in this group to date. However, evidence from behavioral and ecological perspectives supports the

importance of venom economization in spiders. Compelling evidence also establishes that spiders typically deploy their venom in an optimal manner, as documented for four major strategies. Many spiders are highly selective in their use of venom; many appear capable of metering their venom by number of doses or quantity within a single dose; at least some and perhaps many may be capable of modulating the composition of their venom gland secretions during successive episodes of deployment; and many are capable of selectively targeting the delivery of their venom to a particularly vulnerable region of their target, or to a relatively safe region to avoid retaliatory injury.

Although these four behavioral strategies of spiders are supported by the current body of research, the behaviors of only a very small proportion of the 45,000-plus species (Natural History Museum Bern 2015) have been studied thus far. By contrast, researchers have generated substantial details on other aspects of venom (e.g., biochemistry, pharmacology, toxicology) for a much greater diversity of spiders (Nentwig and Kuhn-Nentwig 2013). Again, researchers have to a large extent neglected to study the behavioral roles and usage of venom. Investigators need not only to examine venom use for a greater variety of species but also to identify possible sex differences and ontogenetic changes. Further studies of economization and optimization in spiders will shed light on the many opportunities and constraints associated with venom deployment that are afforded by the diverse trophic specializations within this group.

The possible role of cognition in spider venom deployment will no doubt receive further attention. Unfortunately, authors continue to differ in how they define cognition, which ranges from more general concepts, including any use of information from the environment, to the need for higher-order processing (beyond the normal stimulus-response pathway), such as cognitive maps, planning, and concept learning (e.g., Menzel 2013; Perry et al. 2013; Shettleworth 2013). In part due to their relative structural and neurological simplicity, spiders have proven to be useful models for exploring the features of behavioral decision making and cognition (Jackson and Cross 2011). Spiders exhibit complex, flexible behavior that sometimes closely parallels that of much bigger animals. Although better documented in larger animals, spiders are able to decide between different options in reference to the expected outcome of potential actions. Venom deployment by spiders may well satisfy some or many definitions of cognition, but more careful assessment is required to rule out simpler explanations.

The venom delivery system and venom deployment strategies of spiders invite comparisons to other animal groups. Many venomous taxa possess a single venom delivery system, which is often placed posteriorly on the body (e.g., the stinger of scorpions and hymenopterans). Spiders share more in common with snakes, however, in having a paired venom delivery apparatus placed anteriorly, which includes two chelicerae with fangs. In both snakes and spiders, the right and left venom delivery systems can function independently, which creates unique opportunities for prey capture and handling (e.g., backward stabbing by atractaspidid snakes) and fang repositioning during venom deployment (Hayes 2008). At least one group of spiders

delivers venom into prey with a single fang at a time (Rezac et al. 2008), but the functional aspects of two independent venom delivery systems warrant further consideration. Some animals, including certain groups of ants, wasps, scorpions, and snakes, are capable of modulating the mechanism of venom expulsion, delivering toxins via either biting or spraying. One araneomorph group, the spitting spiders, is similarly capable of both biting and spitting. However, the content of the spider secretions differs, with spits comprised mostly or entirely of adhesive, glue-like strands produced in the anterior portion of the venom gland, which immediately immobilize the prey, and subsequent bites delivering venom produced in the posterior portion of the venom gland, which further incapacitates or kills the prey (Zobel-Thropp et al. 2014). Stimuli relied on for decisions regarding venom use also appear to differ among venomous animals. Whereas many snakes bite and then immediately release their prey, which presumably requires a decision on the snake's part prior to attack (Hayes et al. 2002), spiders generally maintain contact with their prey during and/or subsequent to envenomation and evaluate the optimal venom dose based on the prey's struggle. However, considering the apparent ability to perceive venom availability (Wullschleger and Nentwig 2002) and threat level (Nelsen et al. 2014a) in ways that influence venom use, spiders may also have the ability to decide venom dose prior to attack. Ant-eating spiders of the genus *Zodarion* offer an excellent model to study this possibility, as they typically deliver a single bite or two to their giant, dangerous prey and then wait for envenomation to take effect (Pekar et al. 2014). Finally, some groups of spiders share with hymenopteran insects the capacity to deliver venom as a social group. In doing so, these animals are able to procure larger prey items than otherwise possible and are better able to defend themselves against attack (e.g., Campon 2007; Grinsted et al. 2013).

Behavioral opportunities and constraints related to venom deployment are associated with morphological and biochemical traits that act in concert to achieve a desired outcome (Pekar and Toft 2014). The capacity of spiders to meter their venom, or to target vulnerable body regions of a target animal, can presumably influence selection on a number of traits, and vice versa. Examples include size and shape of structures related to venom storage and delivery; neurological complexity to support decision making; and venom composition, since different peptides and proteins may be more effective when delivered to or near ganglia in the head or thorax compared to delivery into the abdomen. Spiders within a single genus (*Dysdera*) specialized to feed on woodlice (Crustacea, Oniscoidea) illustrate how multiple solutions can evolve in response to some of these complex relationships (Rezac et al. 2008). Species having elongate chelicerae insert a single fang into the soft ventral side of their woodlouse prey and place the other chelicera on the dorsal side of their prey; species with dorsally concave chelicerae quickly tuck their chelicerae under their prey to bite the ventral surface; and species with flattened chelicerae insert their chelicerae between the sclerites into the armor of the woodlouse. Future studies with diverse spider groups will offer further insights on how venom deployment relates to other non-behavioral traits.

Cross-References

► Evolutionary Context of Venom in Animals
► Venom as a Component of External Immune Defense in Hymenoptera

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Independent Origins of Scorpion Toxins Affecting Potassium and Sodium Channels

9

Shangfei Zhang, Bin Gao, and Shunyi Zhu

Abstract

Peptide neurotoxins targeting sodium (Na^+) and potassium (K^+) channels are two major components of scorpion venom for capturing prey (e.g., insects) and deterring competitors (e.g., small mammals). Although a great amount of information in terms of their sequences, structures and pharmacological functions is available currently, the origin of these toxins remains unsolved. Based on the genomic organization and three-dimensional structure similarities together with close functional relatedness, it has been proposed that these two types of molecules could arise from a common ancestor. However, recent studies have provided convincing experimental evidence in favor of their independent origins, in which an ancestral K^+ channel toxin firstly evolved from an antibacterial insect defensin-like molecule via a small deletion of the amino-terminal loop (n-loop) to remove steric hindrance between peptide-channel interaction whereas scorpion Na^+ channel toxins originated from an antifungal drosomycin-like ancestor through the insertion of a small amino-terminal turn and the extension of a carboxyl-terminal tail to reach a new receptor region on the channels, in line with the discovery that drosomycin can bind to the *Drosophila*'s own Na^+ channels. These studies highlight the importance of insertion/deletion (indel) mutations in toxic origin from ancestral scaffolds of physiological functions.

Keywords

Scorpion toxin • Voltage-gated ion channel • Drosomycin • Insect defensin • Evolution

S. Zhang • B. Gao • S. Zhu (✉)
Group of Peptide Biology and Evolution, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
e-mail: zhangshangfei@ioz.ac.cn; gaob@ioz.ac.cn; zhusy@ioz.ac.cn

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_12

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Introduction

Scorpions are one of the most ancient arthropods and have existed on earth for more than 400 million years without obvious morphological change (Possani et al. 1999). They developed polypeptide-rich venom as weapon for predation and defense. These toxic peptides impair functions of a variety of ion channels (Na^+, K^+, Cl^- and Ca^{2+}) present in excitable membranes via interacting with their voltage-sensor domain (VSD) or the channel pore (Zhu et al. 2005; Wang et al. 2011; Zhang et al. 2011; Banerjee et al. 2013). Among them, toxins affecting Na^+ and K^+ channels are the two most abundant peptide components in the venom. These neurotoxins comprise about 28–76 amino acids with two to four disulfide bridges and the majority of them adopt a cysteine-stabilized α-helical and β-sheet (CSαβ) fold.

Although a great amount of information in terms of their sequences, structures and pharmacological functions is available currently, the origin of these toxins remains an unsolved issue. Similarities in the genomic organization and three-dimensional structure together with close functional relatedness appear to support an opinion of common origin for these two types of molecules (Froy et al. 1999). In particular, it was proposed that the scorpion short-chain K_v channel toxins might be evolved from a long-chain Na_v channel toxin via genetic "CC" deletion (Céard et al. 2001). Unfortunately, this proposal is only based on their isolated cDNA sequences and thus genetic polymorphism of the Na_v channel toxin gene cannot be ruled out. By using experimental evolution studies, we provide convincing evidence in favor of their independent origins, in which the ancestor of K^+ channel toxins is traced to an antibacterial insect defensin-like molecule via evolutionary deletion of a loop to remove steric hindrance between the peptide-channel interaction (Zhu et al. 2014) and an ancestor similar to the antifungal drosomycin is proposed to have evolved into scorpion toxins affecting Na^+

channels via assembly of a functional subdomain on a conserved scaffold to target new receptor site on the channels (Zhu et al. 2010b). This work summarizes current progress on the origin and evolution of these two types of neurotoxins.

Classification and Structures of Scorpion Potassium Channel Toxins

K^+ channels are the most diverse type of ion channels with extensive distribution in nearly all cells of prokaryotic and eukaryotic organisms. They form K^+-selective pores spanning cellular membranes and regulate a wide variety of physiological functions of excitable and non-excitable cells, such as regulation of action potential of neurons and muscles as well as secretion of hormones. Voltage-gated K^+ channels (K_v) are homo-tetramer comprising four α-subunits, each containing six transmembrane helices (S1-S6). The S1 to S4 helices comprise the VSD and S5 to S6 constitute the pore domain for specific K^+ ion conduction. Apart from α-subunits, K^+ channel complex usually contain additional β-subunits for modulation of the kinetics and voltage dependence of the α-subunits and the T1 linker domain that restricts K_v channel subunit heteromultimerization (Chen et al. 2010). Given key physiological functions, K^+ channels have been frequently selected as targets of a diversity of venomous animals, in which the pore domain is a major site for toxin binding to inhibit the passage of K^+ ions (Banerjee et al. 2013).

Scorpion venom is a rich source of peptides targeting various types of K^+ channels (KTxs). To date, about 120 KTxs have been characterized via a combination of molecular and biochemical techniques (Rodriguez de la Vega et al. 2003). On the basis of sequence similarity as well as phylogenetic relationship, KTxs have been divided into three major groups: α, β, and γ, which all adopt a conserved CSαβ fold (Tytgat et al. 1999): (1) Alpha-KTxs are the largest group that contains at least 26 different subfamilies. They are short-chain peptides composed of 28–45 residues with three to four disulfide bridges and block several types of K^+ channels, such as voltage-gated *Shaker*-related K^+ channels (K_v), *ether-a-go-go*-related (ERG) K^+ channels, Ca^{2+}-activated K^+ channels of high (BK), intermediate (IK) and small (SK) conductance (Rodriguez de la Vega and Possani 2004). Figure 1a presents a representative structure of α-KTxs (Fig. 1a); (2) Beta-KTxs are a group of long-chain toxins composed of 50–75 amino acids cross-linked by three disulfide bridges. In comparison with α-KTxs, all the β-KTx members possess an N-terminal extension that folds into α-helical conformation and thus named N-terminal helix domain (NHD) (Fig. 1b) (Zhu et al. 2010a). Their carboxyl-terminal domain is similar to α-KTxs and defensins from multicellular organisms, called C-terminal CSαβ domain (CCD domain). Functionally, some β-KTxs exhibit dual activities as K_v channel blockers and microbicidel agents; (3) Gamma-KTxs consist of 36–43 amino acids with three or four disulfide bridges. These peptides specifically affect the ERG family of K^+ channels and have an additional helix in their N-terminus (Frenal et al. 2004).

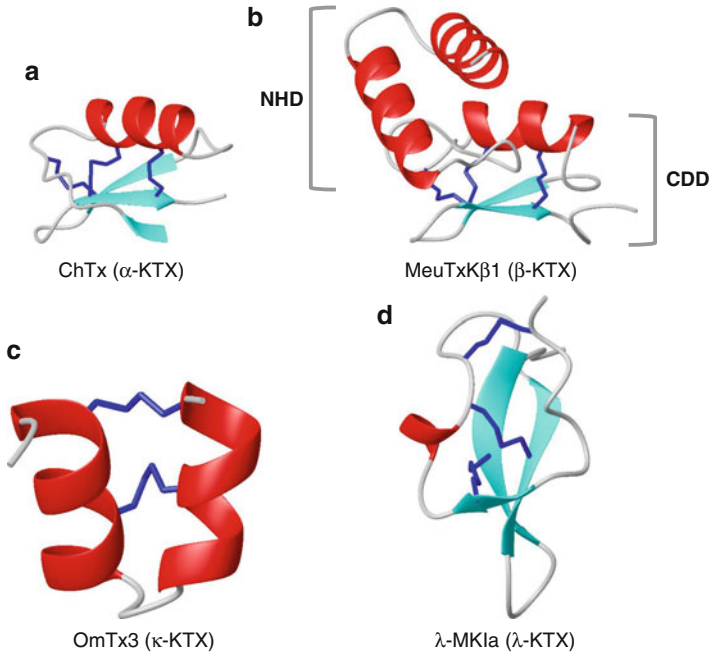

Fig. 1 The fold diversity of scorpion toxins affecting K^+ channels. (**a**). CoTx1 (α-KTx) (pdb entry 1PJV); (**b**) MeuTXKβ1 (β-KTx) (Zhu et al. 2010a). NHD, N-terminal α-helical domain. CDD, C-terminal defensin domain; (**c**) OmTx3 (κ-KTx) (pdb entry 1WQE); (**d**) λ-MKIa (λ-KTx) (Gao et al. 2013). Ribbon models are generated by MolMol (http://www.gunda.hu/mol2mol/index.html)

In addition to these major groups, scorpion venom also contains several minor KTx components with different folds (Gao et al. 2013), including: (1) κ-KTxs are short peptides with extremely low sequence similarity to other families. Members in this family adopt an uncommon cysteine-stabilized α-helix-loop-helix (CSα/α) scaffold with two disulfide bridges (Fig. 1c). OmTx1 to OmTx3 are three κ-KTx members purified from *Opisthacanthus madagascariehsis* with inhibitory effect on $K_v1.1$, $K_v1.2$, and $K_v1.3$ channels (Chagot et al. 2005); (2) λ-KTxs. Toxins in the λ-KTx family adopt an inhibitor cysteine knot (ICK) fold composed of a three-strand antiparallel *β*-sheet and a 3_{10}-helix (Fig. 1d). *λ*-MeuKTx-1 is a representative of this family and the first number isolated from the scorpion venom of *Mesobuthus eupeus*. *λ*-MeuKTx-1 specially blocks the *Drosophila Shaker* K^+ channel (Gao et al. 2013); (3) The Kunitz-type K^+ channel toxin family. All members share a common cysteine pattern and act as specific inhibitors of K_v channels (Chen et al. 2012); (4) κ-BUTX-Tt2b and Ts16. These two peptides are weak inhibitors of K_v channels isolated from the *Tityus trivittatus* venom and have a similar cysteine spacing to α-KTxs but unconventional disulfide bridge pattern. They fold into an uncommon cysteine-stabilized helix-loop-helix (CSαα) structure cross-linked by three disulfide bridges (Saucedo et al. 2012).

Interactions Between Scorpion Toxins and Potassium Channels

In line with their molecular diversity, scorpion KTxs employ multiple functional regions to bind to different types of K^+ channels, which can be summarized as follows: (1) *Alpha-helix*. Toxins derived from the fifth subfamily of α-KTxs, such as BmP05 and P05, block SK channels mainly by α-helical residues, including a positively charged area (two Arg residues in the RRCQ motif) (Wu et al. 2002). The γ-KTx, BeKm1, also uses its α-helix to block the ERG channels where four amino acid residues (Tyr11, Phe14, Lys18, Arg20) are located on the interface of the toxin-channel complex (Korolkova et al. 2002). Two basic residues (CKKX, CKXK, or CXKKX) in the α-helix of several α-KTxs constitute a "hot spot" for the pore blockade of the ERG channels (Abdel-Mlttaleb et al. 2008); (2) *Beta-strands*. It is known that a conserved dyad motif constitutes a minimum functional unit of many toxins to interact with the channel pore, which is composed of a basic Lys located in a β-strand together with a neighboring aromatic (Phe and Tyr) or hydrophobic (Leu) residue within about 7 Å of distance (Dauplais et al. 1997; Rodriguez de la Vega et al. 2003). The dyad has been convergently evolved in many toxins from different venomous species, such as sea anemones, snakes and cone snails. The same Lys in combination with an Asn located between two β-strands was also found functionally important, in which the Asn is proposed to bind to an acidic Asp in the pore helix via H-hydrogen interaction while Lys directly inserts into the channel to interact with Tyr on the filter region of the channel (Lange et al. 2006; Zhu et al. 2014); (3) *C-tail*. Hg1, a Kunitz-type toxin from the Mexican scorpion *Hadrurus gertschi*, was found to interact with K^+ channels by its C-terminal region (Chen et al. 2012); (4) *Multiple domains*. Pi1, a scorpion toxin isolated from the venom of *Pandinus imperator*, uses four residues from different domains of the toxin to assemble a basic ring interacting with $K_v1.2$ (Mouhat et al. 2004). In ErgTx1, a γ-KTx purified from the venom of the scorpion *Centruroides noxius*, four residues (Tyr14, Tyr17, Met35 and Phe37) derived from different secondary structural elements constitute a hydrophobic patch to bind to the hERG1 K^+ channel (Frenal et al. 2004; Jimenez-Vargas et al. 2012).

The dyad-mediated toxin-channel interaction is the most common mode between α-KTxs and K_v channels. Thanks to the work of Banerjee et al., the first experimental complex structure between a KTx and a channel pore is available currently. This crystal structure includes a chimeric K_v channel and the scorpion toxin CTX from *Leiurus quinquestriatus*. In this complex, CTX binds into the extracellular side of the pore of the channel in a key and lock manner, in which Lys27 in CTX inserts into the pore to make direct contact with the backbone carbonyl oxygen of Tyr445 in the paddle chimera (Banerjee et al. 2013). KTX, an α-KTx of 37-residues originally isolated from the venom of the scorpion *Androctonus mauretanicus mauretanicus*, uses similar residues CTX to bind a channel pore. However, it is remarkably different from the CTX's rigid mode, KTX's binding induces conformational changes in the selectivity filter of the pore of a K^+ channel chimera (KcsA-Kv1.3), as revealed by a solid-state NMR study (Lange et al. 2006).

From Defensins to Potassium Channel-Targeted Neurotoxins: Evolutionary Deletion of n-Loop

The study of the origin of KTxs is a great challenge as their enormous sequence diversity and multiple action modes. More recently, based on experimental evolution strategy guided by the concept of evolutionary intermediate, Zhu et al. have provided convincing evidence in favor of the origin of α-KTxs from ancestral CSαβ-type antibacterial defensins (Zhu et al. 2014). This kind of defensins constitute essential innate immunity components of insects and other arthropods in fighting against microbial infection. In addition to their structural similarity, scorpion α-KTxs and insect defensins are also functionally related, both involved in either defense against competitors or invasive microbes by disrupting their cellular membrane functions. To establish evolutionary link between these two types of molecules, the authors firstly defined "Scorpion Toxin Signature" (STS) comprising the evolutionarily conserved and structurally/functionally important residues, described as "C. . .CXXXC. . .KCXN. . .CXC" (C, Cys; X, any non-cysteine amino acid; K, Lys; N, Asn) (Zhu et al. 2014). By using the STS, they searched for defensins from six insect Orders to find potential evolutionary intermediates (i.e., defensins containing the STS) for experimental study. As a result, eight classical insect-type defensins (CITDs) were identified to contain the STS, which all are restricted to two venomous insect Orders (Hemipteran and Hymenopteran). Of them, navidefensin2-2 was found to recruit into the venom gland of *Nasonia vitripennis*, suggesting its evolutionary potential in developing K_v channel-targeted neurotoxins.

Structurally, these CITDs have a conformationally flexible amino-terminal loop (n-loop) that is lacking in α-KTxs. This region exhibits variable sizes among different members, indicating its genetic variability in tolerance of indel mutations. More importantly, when a CITD was placed on the interface of toxin-channel interaction according to the mode of α-KTxs, this loop gave rise to severe steric hindrance. Based on these findings, it was proposed that the deletion of the n-loop of an evolutionary intermediate to remove the steric hindrance could be a key evolutionary event mediating the emergence of α-KTxs. To validate this deduction, the authors deleted the n-loop of navidefensin2-2 and named this new peptide navitoxin (Fig. 2) (Zhu et al. 2014). As expected, navitoxin folds into an α-KTx's structure and obtains capability in blocking several subtypes of K_v channels with nanomolar affinity accompanying the loss or reduction of antibacterial activity due to the deletion of the functional n-loop. Similar to α-KTxs, navitoxin also uses two key residues in the STS (Lys21 and Asn24) to interact with the channel pore (Fig. 2) (Zhu et al. 2014).

The removal of steric hindrance of a venom CITD for interacting with the K_v channel via small loop deletion induces a switch from antibacterial function to K_v channel blockade, providing key functional evidence for their evolutionary relationship (Zhu et al. 2014). This is further strengthened by the action mode similarity between the CITD-derived peptide and α-KTxs, in which the ancestral form of CITDs firstly evolved a Lys-Asn motif in venomous animals and then the loop was deleted in the scorpion lineage to evolve K_v channel-targeted toxins (Fig. 2).

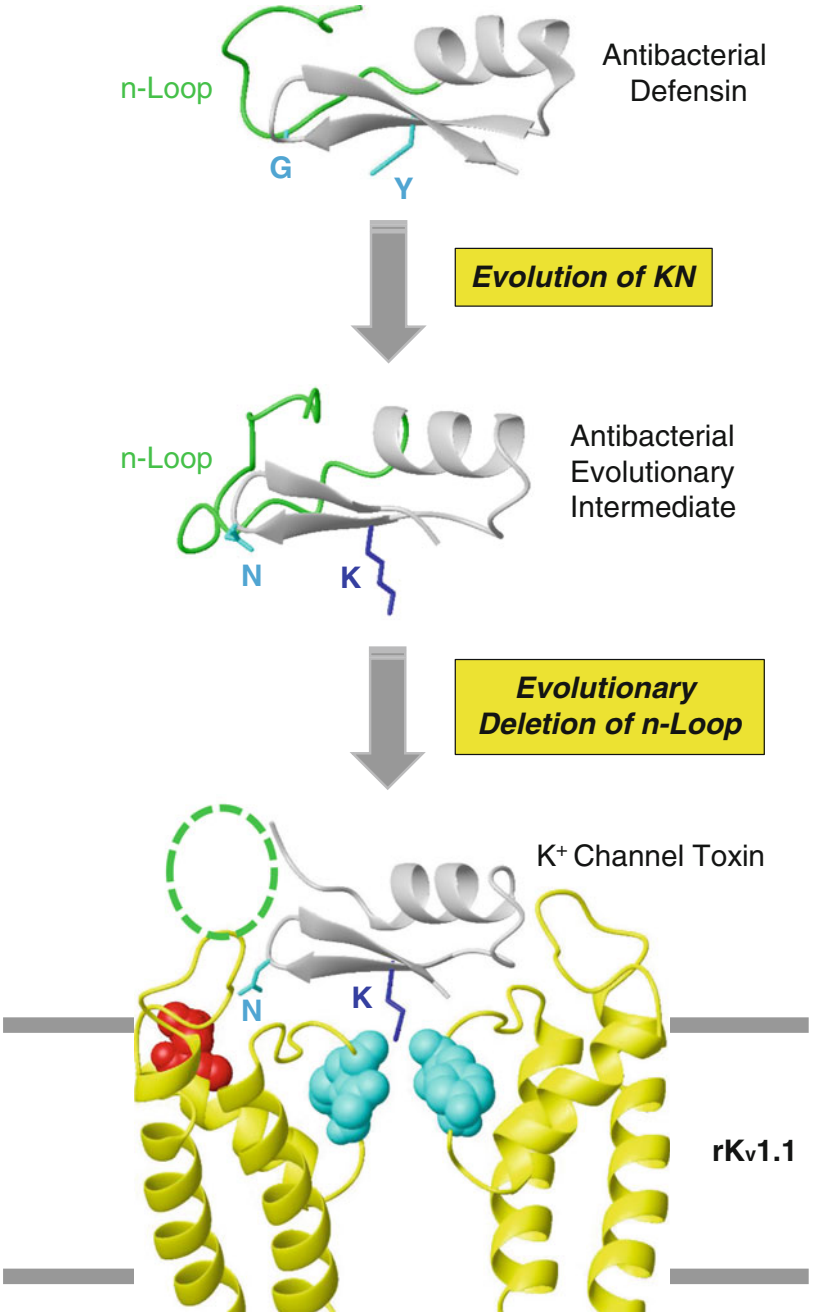

Fig. 2 The origin of a scorpion K^+ channel toxin from an antibacterial defensin. The antibacterial defensin is sapecin (pdb entry 1L4V) and its two residues corresponding to the KN motif shown. The evolutionary intermediate used here is navidefensin2-2 and the K^+ channel toxin is navitoxin (Zhu

Considering extensive distribution of the α-KTxs in many scorpion species, it is reasonable to infer that they represent the earliest components of KTxs. Subsequent accelerated substitutions at key sites expand their targets to other types of K^+ channels, such as ERG and SK channels. BmTx3, an α-KTx isolated from the venom of *Mesobuthus martensii*, represents such an example (Huys et al. 2004). This toxin has two functional surfaces acting on K_v and ERG channels, respectively, and is considered as an intermediate between α-KTxs and γ-KTxs. In addition to evolutionary divergence from a conserved scaffold, the expansion of scorpion KTx arsenal may also occur through evolutionary convergence to recruit endogenous body proteins with various folds into the venom. A specific example is that toxins from different families (e.g., λ-KTxs and κ-KTxs) convergently developed a functional dyad, initially recognized in α-KTxs (Dauplais et al. 1997; Rodriguez de la Vega et al. 2003), to target K_v channels.

Classification and Structures of Scorpion Sodium Channel Toxins

Scorpion toxins affecting voltage-gated Na^+ (Na_v) channels (ScNaTxs) are major toxic components of the venom with lethal effect on both insects and mammals. They are polypeptides of 61–76 residues typically with four disulfide bridges (Possani et al. 1999). ScNaTxs are divided into two distinct pharmacological classes: α-toxins that slow the inactivation process of Na^+ currents to prolong the action potential by binding to the receptor site 3 of Na_v channels; and β-toxins that shift the voltage-dependent activation toward more hyperpolarizing potentials by binding to receptor site 4. According to their preference for insect and mammalian Na_v channels, the α-toxin group can be further divided into three distinct subgroups (Bosmans and Tytgat 2007): (1) Classical anti-mammalian toxins (e.g., AaHII) that are highly active on mammalian Na_v channels; (2) Anti-insect toxins (e.g., Lqq3) that strongly affects insect Na_v channels; (3) Alpha-like toxins (e.g., Lqh3) that are highly toxic to both mammalians and insects. Based on the same criterion, the scorpion β-toxin group is also further divided into these three subgroups: (1) Classical anti-mammalian β-toxins (e.g., Cn2) that are exclusively found in scorpions of the genus *Centruroides* and highly toxic to mammals; 2) Anti-insect β-toxins, including depressant toxins (e.g., LqhIT2) that induce flaccid paralysis and excitatory toxins (e.g., *Bj*-xTRIT) that induce contraction paralysis in fly larvae; (3) Beta-like toxins highly active on both insect and mammalian Na_v channels (e.g., Ts1) (Possani et al. 1999).

Fig. 2 (continued) et al. 2014). *Dotted circle* indicates the removal of steric hindrance between the n-loop and the turret region of the channel pore. KN, Lys and Asn, two key functional residues belonging to the STS (Zhu et al. 2014). Amino acids in the sphere model are key channel residues involved in toxin binding

Despite remarkable pharmacological diversification associated with differential phyletic selectivity, these toxic molecules overall adopt a conserved three-dimensional structure, composed of a cysteine-stabilized α-helical and β-sheet (CSαβ) molecular scaffold and a perpendicular NC-domain comprising an amino-terminal turn and a carboxyl-terminal tail (Fig. 3). The CSαβ scaffold is shared by other scorpion toxins affecting K^+, Cl^- and Ca^{2+} channels and is even present in an

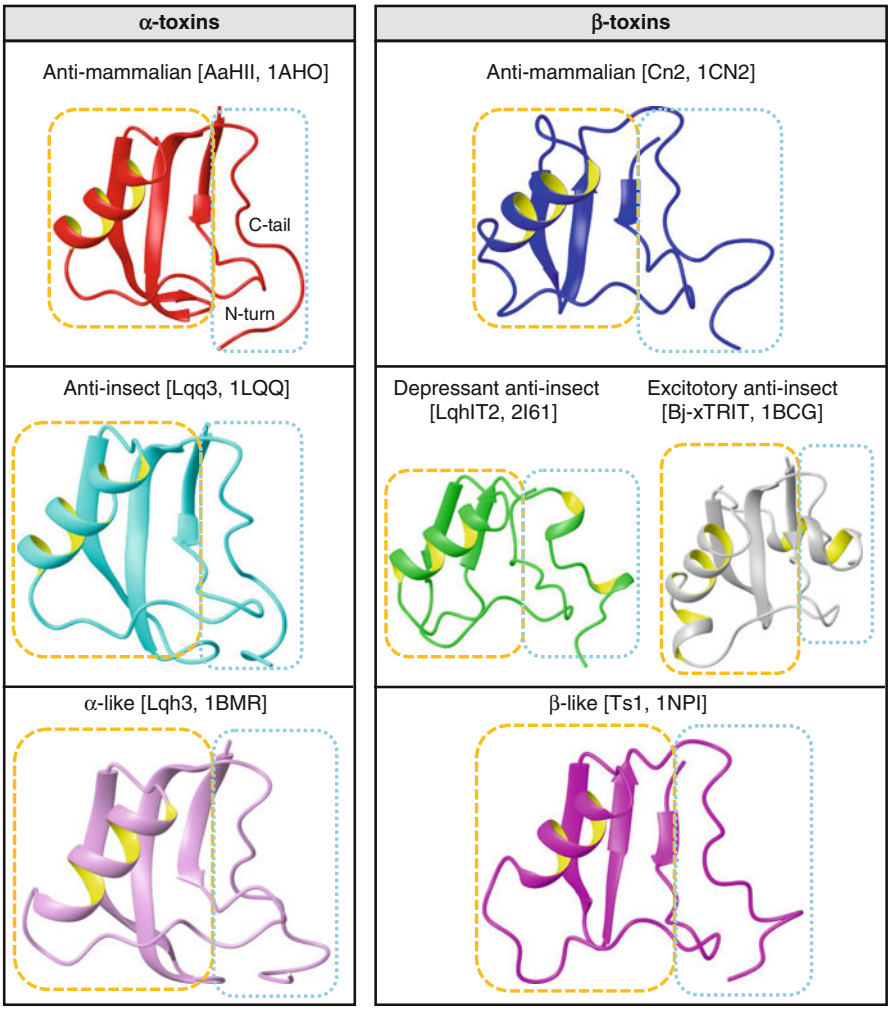

Fig. 3 Representative structures of scorpion α-toxins and β-toxins. Toxin names and their pdb entry numbers are provided in *brackets* and each pharmacological subgroup is shown in different colors. The core- and NC-domain are boxed in *orange* and *light blue*, respectively

array of polypeptides with diverse origins and biological functions, but the NC-domain appears to be unique to scorpion Na_v channel toxins (Zhu et al. 2005).

Functional Domains of Scorpion Sodium Channel Toxins

Previous extensive mutational studies have indicated that ScNaTxs from different pharmacological subgroups employ a similar strategy to assemble their functional surfaces into two distinct subdomains: (1) A core domain that is conserved across all specific pharmacological subgroups and contains the so-called pharmacophore; (2) A variable domain that is most likely determinant of toxin specificity on different Na_v channel subtypes (Gurevitz 2012). For the α-toxins, there exists a common bipartite bioactive surface where the core domain is composed of short loops connecting the conserved secondary structure elements of the molecules, and the NC-domain formed by a five-residue N-turn and a C-tail. Similar active sites in the core domain of Lqh2, Lqh3 and LqhαIT provide structural basis for different toxins binding to the same site in Na_v channels (Gurevitz 2012). By contrast, the difference in the shape of NC-domain among members has been considered to be a factor relevant to the selectivity of α-toxins. This appears true in that in mammal-specific α-toxins, the N-turn is more mobile and it moves with the C-tail in a concerted manner with respect to the core module, but this has not been observed in insect α-toxins (Chugunov et al. 2013).

For the β-toxins, their functional surfaces are also composed of two parts: one situated in the α-helix and its vicinity with a common "pharmacophore"; and another differing among subgroups that determines the selectivity of β-toxins (Gurevitz 2012). The pharmacophore is formed primarily by a negatively charged residue (E^{30} in *Bj*-xtrIT, E^{24} in LqhIT2, E^{28} in Css4, and E^{26} in Lqhβ1) flanked by hydrophobic residues (Gurevitz 2012). While this common feature is related to activity, amino acids in variable domains seem crucial for the selectivity of toxins. For example, the β2-β3 loop in Css4; the C-terminal region in *Bj*-xTRIT; and residues forming a hydrophobic bed and the C-terminal region (especially R^{58}) in LqhIT2, are associated with preference of the toxins for insect Na_v channels (Gurevitz 2012).

Two Distinct Sodium Channel Receptor Sites for Toxin Binding

The Na_v channels are transmembrane protein complexes mainly comprising one pore-forming α-subunit and one or two smaller auxiliary β-subunits (β1-β4) in mammals, or TipE in insects, which modulate the kinetics and voltage dependence of channel gating (Catterall et al. 2005). The α-subunit constitutes an essential functional unit of the channel and usually contains more than 2,000 amino acids with four highly homologous structural domains (DI to DIV), each of which includes six α-helical segments (S1-S6) long enough to cross the membrane and a reentrant

pore loop (P) between S5 and S6 (Fig. 4a). When viewed from the outside, DI to DIV are arranged in a clockwise pattern (Fig. 4b). The first four-helix (S1-S4) bundle forms a modular VSD to initiate channel activation, and S5-S6 and the intervening P-loop form the pore domain allowing Na^+ to across the membrane. The conformational change of the VSD of one domain in response to depolarization can be transmitted to the pore module of the neighboring domain (Zhang et al. 2011). The S4 segment is a voltage sensor of the channels with four to seven conserved positively charged residues separated by two hydrophobic residues (Catterall 2000). They move outward upon depolarization and transport the gating charges from inner membrane to out membrane (Wang et al. 2011; Zhang et al. 2011).

A variety of peptide toxins exert effect on Na_v channels via binding to specific receptor sites (Zlotkin 1999), among which site 3 is targeted by scorpion α-toxins and site 4 by scorpion β-toxins. With the exception of the Na^+ channel blocker Cn11 isolated from the venom of *Centruroides noxius* (Ramirez-Dominguez et al. 2002), all ScNaTxs affect gating of the channels (Possani et al. 1999). Site 3 is formed by residues in the extracellular linker in DI (SS2-S6) and the extracellular linker connecting segments S1-S2 and S3-S4 in DIV; and site 4 consists of residues in the SS2-S6 loop in DIII and S1-S2 and S3-S4 loops in DII (Zhang et al. 2011) (Fig. 4c). Structural studies indicate that a S5-S6 loop in a domain is in close proximity to S1-S2 and S3-S4 loops in its adjacent domain (Payandeh et al. 2011).

The division of bioactive surfaces of ScNaTxs into two distinct domains mirrors their receptor site division, as supported by two recent toxin-channel complex models built based on a series of mutational data both from toxins and channels (Lqh2-rNa_v1.2 and Css4-rNa_v1.2) (Wang et al. 2011; Zhang et al. 2011; Zhang et al. 2012). In these models, although sites 3 and 4 are located on different domains of Na_v channels, S1-S2 and S3-S4 loops from one domain are required for binding to ScNaTxs via the core-domain of these toxins; whereas SS2-S6 loop derived from the adjacent domain may contact the NC-domain of toxins and play a secondary role.

Sequence and Structural Similarity between Drosomycin and Scorpion Sodium Channel Toxins

Drosomycin is the first inducible antifungal peptide identified in insects, which was initially isolated from *Drosophila melanogaster* (Fehlbaum et al. 1994) and recently found in some ecdysozoans (Zhu and Gao 2014). This cationic peptide of 44 amino acids adopts a compact CSαβ structure composed of one α-helix and an anti-parallel three-stranded β-sheet (Landon et al. 1997). Drosomycin shares significant sequence similarity (>50%) to the core region of scorpion depressant toxins (Zhu et al. 2005; Zhu et al. 2010b). When compared with the depressant toxin LqhIT2, a total of 19 identical residues and five conservative replacements were identified. In particular, six functional residues previously characterized to be important for channel binding of LqhIT2 (Karbat et al. 2007) are also conserved in drosomycin, including L^3, K^8, P^{10}, A^{12}, V^{13} and R^{21} (Fig. 5a). Structural comparison revealed that the core

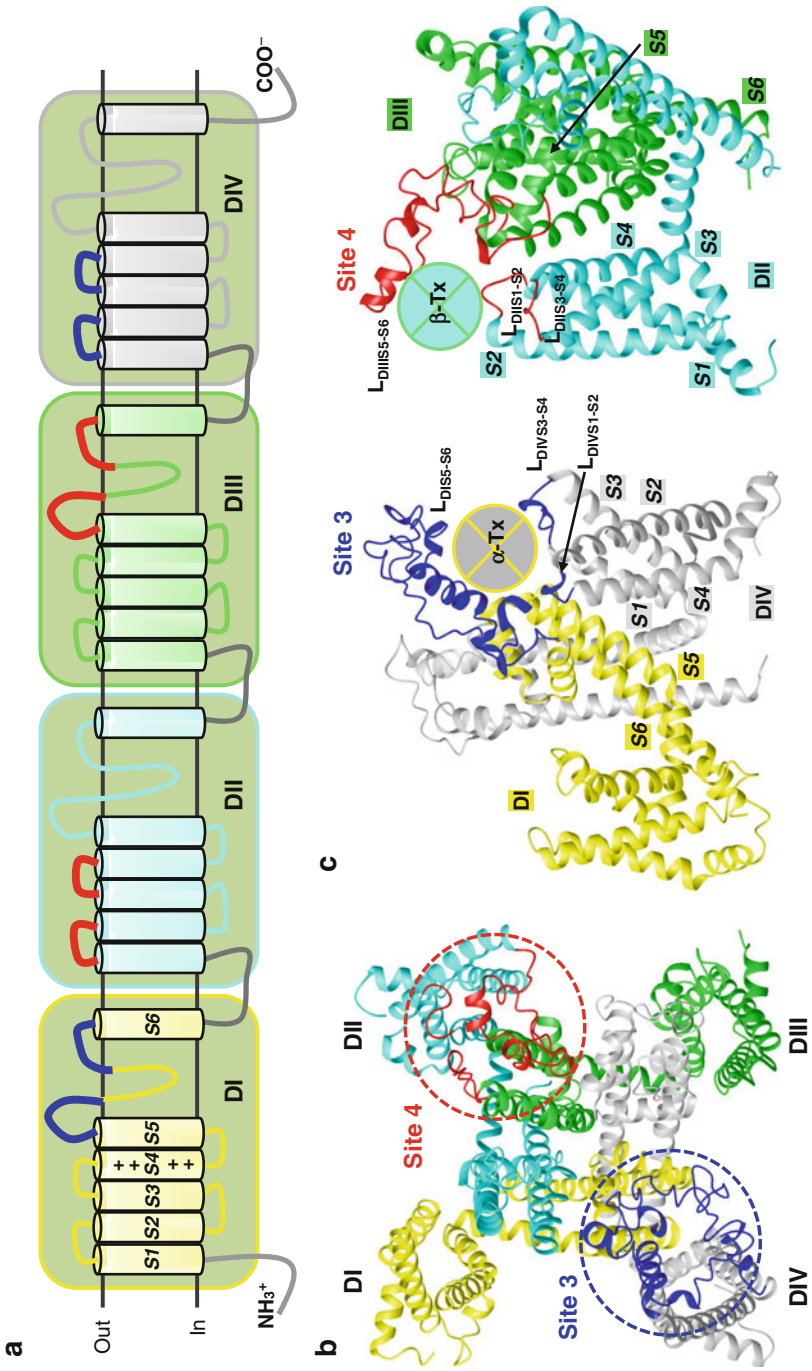

Fig. 4 (continued)

of LqhIT2 accurately matches drosomycin but its extra NC-domain is lacking in drosomycin (Fig. 5b). Sequence and structural similarities along with functional relatedness (defense against multicullular organisms) support an orthologous relationship between depressant β-toxins and drosomycins (Zhu et al. 2005).

From Drosomycin to Toxins: Evolutionary Gain of an NC-Domain

To provide functional evidence to establish a reliable evolutionary link, the NC-domain of a depressant scorpion toxin BmKITc was grafted onto the scaffold of drosomycin and the engineered chimeric peptide, termed drosotoxin, changes its target from fungi to rat ion channels (Zhu et al. 2010b). This discovery is further strengthened by two remarkable observations: (1) The deletion of N- and C-terminal sequences of a scorpion β-toxin restored its antifungal activity (Cohen et al. 2009); (2) Drosomycin can bind to its own Na_v channel ($DmNa_v1$) to induce a conformational change (Cohen et al. 2009).

The Na_v channel binding feature and high sequence and structural similarities to the depressant β-toxins suggest that this Na_v channel-targeted molecule might bind to site 4 of $DmNa_v1$ in a manner similar to LqhIT2. The constructed complex models of drosomycin or LqhIT2 with the $DmNa_v1$ site 4 in reference with the structure of Css4-$rNa_v1.2$ (Zhang et al. 2011; Zhang et al. 2012) indicate that drosomycin interacts with the loops connecting S1-S2 and S3-S4 in DII via three hydrophobic residues (P^{10}, A^{12} and V^{13}) which are structurally equivalent to A^{13}, L^{15} and I^{16} in the interface of LqhIT2 and the VSD of $DmNa_v1$ (Karbat et al. 2007). Different from drosomycin, LqhIT2 also binds to the SS2-S6 loop in DIII via its NC-domain (Fig. 6).

Taken together, it is becoming clear that evolutionary gain of an NC-domain on a Na_v channel-targeted ancestral scaffold (drosomycin) represents a key event that mediates the emergence of ScNaTxs through extending its interacting region around the two loops of DII to the SS2-S6 loop in DIII (Fig. 6). In other words, a drosomycin-like molecule acts as a toxin only when it can simultaneously interact with the two regions of a Na_v channel (the two loops in DII: S1-S4 and the SS2-S6 loop in DIII: S5-S6) after the gain of the NC-domain (Fig. 6). According to the current viewpoint, most of animal toxins are developed from related normal body proteins by gene duplication and subsequent mutations to modify their structure and

Fig. 4 The structure of Na_v channel α-subunit. (**a**) The topology showing four repeating domains (DI-DIV), each consisting of six membrane-spanning segments (S1-S6). S1-S6 segments and their connecting loops are respectively colored in *yellow*, *cyan*, *green*, and *gray*, except the loops comprising sites 3 and 4 that are colored in *blue* and *red*, respectively; (**b**) The model structure of human $Na_v1.7$ (Yang et al. 2012). The color code of four domains is in accordance with that of Fig. 4a; sites 3 and 4 are *circled* in *blue* and *red*, respectively; (**c**) Sites 3 and 4 shown to bind to a toxin, in which three loops ($L_{DIVS1\text{-}S2}$, $L_{DIVS3\text{-}S4}$ and $L_{DIS5\text{-}S6}$) comprising site 3 are marked and colored in *blue* whereas the loops ($L_{DIIS1\text{-}S2}$, $L_{DIIS3\text{-}S4}$ and $L_{DIIIS5\text{-}S6}$) forming site 4 marked and colored in *red*

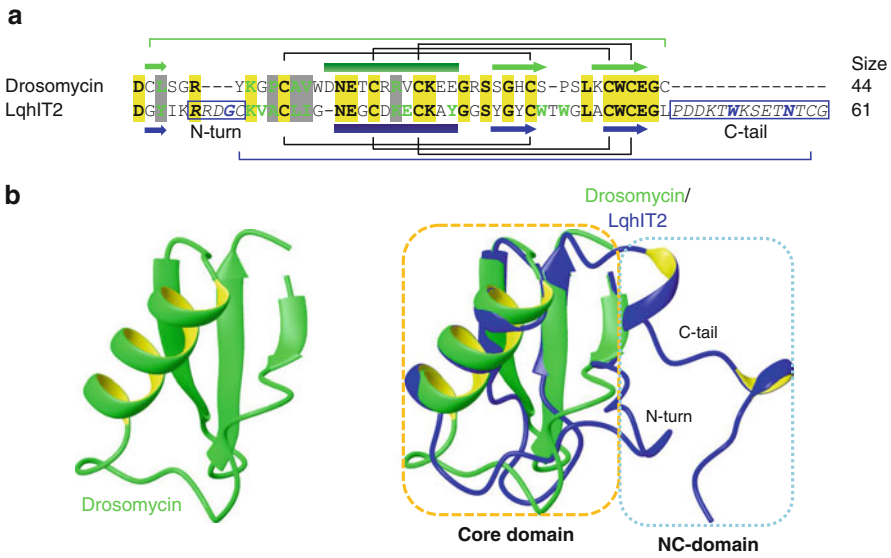

Fig. 5 Sequence and structural similarity between drosomycin and LqhIT2. (**a**) Sequence alignment. Identical residues are shadowed in *yellow* and identical disulfide bridges shown in *black lines*. Amino acid residues identified crucial for activity in LqhIT2 (Karbat et al. 2007) are shown in *green* in the core domain and *blue* in the NC-domain; the structurally equivalent residues to those of LqhIT2 in drosomycin colored in *green* and highlighted in *gray* if they display conservative replacement; (**b**) Structural superimposition of drosomycin (pdb entry 1MYN) with LqhIT2 (pdb entry 2161)

function (Fry 2005). However, it appears that evolution of the first ScNaTx is a result of genetic modification of an ancestral drosomycin-like peptide without gene duplication, as evidenced by the absence of the antifungal peptide in the scorpion genome (Cao et al. 2013b). Froy and Gurevitz hypothesized that an ancestral long-chain Na_v channel toxin first developed into β-like toxins in the New World scorpions and then evolved into α- and β- toxins, including depressant toxins, in the Old World scorpions (Froy and Gurevitz 2003). However, the establishment of evolutionary link between drosomycin and depressant β-toxins suggests that the first ScNaTx evolved should be a depressant β-toxin in the Old World scorpions and subsequent gene duplication combined with speciation generates multiple pharmacological groups.

Drosomycin Binding to Fly Sodium Channels as Endogenous Ligands?

The *Drosophila* fat body, a functional equivalent of the mammalian liver, is a major site of the expression of a series of antimicrobial genes. When *Drosophila* is challenged by fungi, drosomycin is rapidly synthesized in the fat body and secreted

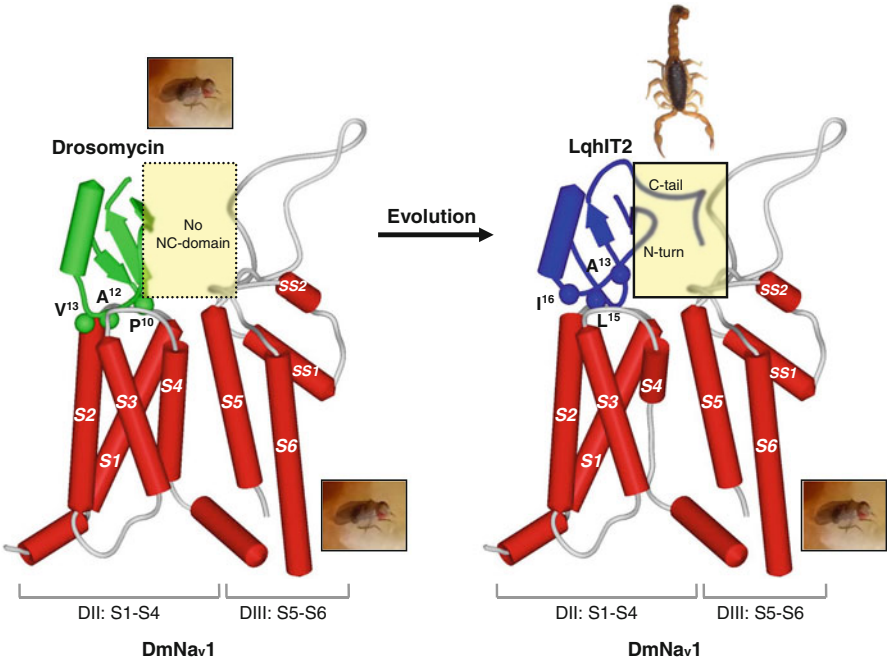

Fig. 6 Structural models in favor of drosomycin and LqhIT2 interacting with a common site on the voltage-sensor domain (VSD) from DII of $DmNa_v1$. Drosomycin is colored in *green* and LqhIT2 in *blue*. The absence and presence of an NC-domain in drosomycin and LqhIT2 are indicated by *dotted* and *solid boxes*, respectively. Three hydrophobic residues conserved between drosomycin and LqhIT2 and hypothesized to be implicated in channel binding are shown in their C_α atoms. The structural models were prepared with WebLab ViewerLite 4.0 (Molecular Simulations Inc.)

into the haemolymph (Fehlbaum et al. 1994). This systemic response is controlled by the Toll signal pathway (Lemaitre et al. 1996). Apart from its antifungal role, the discovery that drosomycin binds to its own Na_v channel gives a clue to other biological functions of this immune molecule. It is known that glia are major contributors to central nervous system immunity in both *Drosophila* and mammals and that their activation is largely dependent on the activity of Na_v channels (e.g., $Na_v1.6$). Moreover, in a recent study, Cao et al. found that *drosomycin* is expressed in neurons and glia of *Drosophila* brain (Cao et al. 2013a). Co-expression of *drosomycin* and *DmNav1* in brain suggests this gene could exert specific effects on brain immunity, presumably acting as an endogenous ligand of $DmNa_v1$ to activate glia-dependent innate immunity. In addition, Na_v channels have been shown to play a vital role in development, aging and neurodegeneration of flies and their adaptation to temperatures (Garber et al. 2012). These discoveries are very attractive as in addition to inducible expression in response to fungal infection, *drosomycin* is also constitutively expressed in three developmental stages of *Drosophila* (i.e., larva, pupa and adult) (Tian et al. 2008). It has also been shown that diapause can lead to increased transcription of *drosomycin* in the absence of infection, and that

drosomycin is a target gene of insulin signaling that controls body size and some life history traits, such as fertility and lifespan (Becker et al. 2010; Kubrak et al. 2014). All these provide evidence supporting other physiological roles of *drosomycin* beyond immunity. Experimental confirmation of Na_v channel ligand function of drosomycin will help elucidate the explicit mechanism behind these biological processes.

Conclusion and Future Directions

In conclusion, there is multidimensional evidence for independent origins of scorpion toxins targeting K^+ and Na^+ channels. This appears to be obviously different from the sea anemone venom K^+ and Na^+ channel toxins which are considered to arise from a common ancestor (Jouiaei et al. 2015). Evidences for a CITD as the ancestor of scorpion KTxs can be found: (1) These toxins share high structural similarity to CITDs but the lack of an n-loop; (2) Only CITDs contain the STS and they all are restrictedly distributed in venomous insects; (3) At least one member was found to recruit into the venom; (4) Conformational flexibility in the n-loop of CITDs is associated with steric hindrance between peptide-channel interaction; (5) Frequent genetic deletion in the n-loop of CITDs; (6) Experimental deletion of a venom-derived CITD gives rise to a toxin with structural, functional and mechanical similarities to scorpion toxins.

Also, the hypothesis that ScNaTxs might originate from an ancestral antifungal drosomycin is supported by the following evidences: (1) At the sequence level, drosomycin and scorpion depressant toxins share >50% similarity and three identical disulfide bridges; (2) The structure of drosomycin exactly corresponds to the core domain of depressant β-toxins; (3) The transfer of the NC-domain of a depressant β-toxin onto the drosomycin scaffold led to the emergence of Na_v channel toxicity whereas a β-toxin with N- and C-terminal sequences deleted exhibited antifungal effect; (4) They both commonly target Na_v channels, although in *Drosophila* such binding could induce an "endogenous ligand" function; (5) Structural studies suggest that drosomycin and depressant β-toxins both use a conserved region to interact with the VSD region of site 4 from DII, and the toxins' NC-domain is close to the adjacent region, SS2-S6 loop in DIII; (6) At the phylogenetic level, the history of drosomycin has been traced to the common ancestor of the Ecdysozoa (Zhu and Gao 2014). Therefore, the evolution of Na_v channel toxins appears to have occurred after the divergence of scorpions from other arthropods. Further investigation of the binding mode between drosomycin and Na^+ channels may provide new insights into the evolutionary mechanism responsible for the origin of toxicity in an ancient scaffold carrying physiological functions.

In recent years, the evolutionary origin of toxins from venomous animals is becoming a hot research topic and the history of many toxins from different species is being uncovered. For example, the origins of shrew and lizard toxins from ancestral serine proteases was achieved via small insertions in several regulatory loops and subsequent accelerated sequence evolution to create new chemical

environment and functional changes (Aminetzach et al. 2009). In addition, it was proposed that two arthropod predators (spider and centipede) convergently employed an ancestral hormone scaffold to develop their venom (Undheim et al. 2015). With more genomes of venomous arthropods sequenced, it is expected that more examples of toxin origin from nontoxic physiological peptides will be uncovered. This will enhance our understanding of toxic peptide evolution and help design novel molecules with specific activity and selectivity.

Cross-References

► Evolutionary Context of Venom in Animals
► Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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Parasitoid Wasps and Their Venoms

10

Mrinalini and John H. Werren

Abstract

Parasitoid wasps are a unique group among venomous organisms. In contrast to the common venom functions of predation and defense, female parasitoid wasps use venom to manipulate the metabolism, development, and behavior of other arthropods for reproductive purposes. This provides a safe environment and nutrition for the next generation of wasps to feed and develop. Parasitoid wasp species diversity is estimated to be between 150,000 and 600,000 species, likely making them the largest group of venomous organisms. They parasitize all orders of Insecta and several taxa from Arachnida. Parasitoids display highly diverse morphologies and parasitic lifestyles. This diversity likely plays a strong role in the adaptive evolution of venom apparatus structures, venom genes, and venom functions. However, parasitoid wasps are underexplored and little represented in toxinology.

This chapter provides a background into evolution of parasitoid wasps and their parasitic lifestyle. The evolution of parasitoid venoms and their functions are discussed, and a comparison of venom functions in two major ecological categories, ectoparasitoids and endoparasitoids, is provided. Expanding on the standard gene duplication and recruitment model of toxin gene evolution, additional mechanisms are proposed. These include co-option, multifunctionalization, alternate splicing, and origins from lateral gene transfers or noncoding DNA. Novel tools such as RNA interference (RNAi) knockdown of parasitoid venom genes, combined with RNA sequencing of envenomated hosts, are proposed for venom function hypothesis testing and hypothesis generation.

Mrinalini (✉)
Department of Biological Sciences, National University of Singapore, Singapore, Singapore
e-mail: dbsmrin@nus.edu.sg; mrinalini.urs@gmail.com

J.H. Werren
Department of Biology, University of Rochester, Rochester, NY, USA

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_2

This chapter also addresses key questions concerning the future directions of parasitoid venom research.

Keywords
Hymenoptera • Insect • Endoparasitoid • Ectoparasitoid • Nasonia • Ovipositor

Contents

Introduction

Parasitoid wasps (also known as parasitic wasps) (Hymenoptera) are a distinctive, but lesser-known, group of venomous organisms. They are typically referred to as parasitoids because although the adult wasps are free-living, their juvenile (larval) developmental stages are completed within or upon other insects or arachnids, eventually killing the "host." In contrast to other venomous animals that use venom for prey capture and/or defense against predators (e.g., snakes, spiders, scorpions, bees, and jellyfish), parasitoid wasps use venom for reproduction and completion of their parasitic life cycle. These wasps parasitize insect or arachnid hosts by injecting venom into them and then laying eggs on or inside the host tissue. Typically, the venom does not immediately kill the host. Rather, it manipulates the host in several ways, such as changing host physiology, metabolism, and behavior and causing paralysis, developmental arrest, and immune suppression (Rivers and Denlinger 1994a, b; Weisel-Eichler et al. 1999; Eberhard 2000; Rivers et al. 2002; Danneels et al. 2013; Martinson et al. 2014; Mrinalini et al. 2014). These changes render the host incapable of performing normal bodily functions and completing normal development. Thus, the wasp ensures a nutritional supply and a safe environment for its larvae, which develop and consume the host.

Despite this evolutionarily innovative use for venom, parasitoid wasps are little acknowledged in toxinology. The often-used definition of venom as "a secretion that disrupts normal physiological or biochemical processes to facilitate feeding or

Fig. 1 Parasitic wasps are usually very small in size. *Nasonia vitripennis*, a model parasitoid species, is seen here on a human fingernail. The small size of parasitic wasps has made venom collection and analysis a challenge until recently (Photo courtesy John (Jack) Werren)

defence by the producing animal" (Fry et al. 2009) does not include the function of parasitic wasp venom. Parasitoid venoms have evolved to promote the growth, development, and the survival of the parasitoid offspring, whereas references to the most common functions of venom are usually foraging and defensive adaptations of the venom producer (Casewell et al. 2013). The biodiversity of parasitoid wasps is estimated at several hundreds of thousands of species (Noyes 2000; Heraty and Gates 2003; Whitfield 2003; Pennacchio and Strand 2006; Heraty 2009; Munro et al. 2011; Noyes 2014). This outnumbers species diversity in every other group of venomous organism and might even outnumber all groups put together. Therefore, the most common function of venom in nature may well be to facilitate the successful completion of the parasitoid life cycle.

The majority of parasitoid wasps are of no danger to humans, which may be one reason they are understudied in toxinology. Added to this is their typically very small size (Fig. 1), which has made venom collection and analysis a challenge until recently. However, these characteristics also make them easier to work with compared to, say, venomous snakes or jellyfish. Parasitoid wasps are ubiquitous around the world, and many species are cosmopolitan in their distribution. Moreover, parasitoid species such as *Nasonia vitripennis* have been established as efficient genetic models due to their short generation time, ease of maintenance in the lab, and interspecies fertility (Werren and Loehlin 2009a, b; Werren et al. 2010). Genotyping and characterization of gene expression are easier, as whole wasps can be used for sequence analysis. Parasitoid wasps are easily genetically manipulated using RNA interference (RNAi) knockdowns, allowing a subtractive approach to venom functional studies via RNAi-mediated downregulation of venom gene expression (Lynch and Desplan 2006; Werren et al. 2009; Colinet et al. 2014b).

Recently, the potential uses of parasitoid venom in pharmacology and agriculture have been recognized (Beckage and Gelman 2004; Zhang et al. 2005; Danneels et al. 2010; Werren et al. 2010; Zhu et al. 2010; Heavner et al. 2013, 2014; Moreau 2013; Colinet et al. 2014a). Given their incredible species diversity and the wide range of parasitic lifestyles, parasitoid wasps are a potential gold mine of novel bioactive peptides. For example, a large proportion of proteins in *N. vitripennis* venom are not found in any other organism (Danneels et al. 2010; de Graaf et al. 2010; Werren et al. 2010). Parasitoid venom functions of targeted manipulations of host immunity, physiology, metabolism, development, and behavior are not found in other venomous organisms (Rivers and Denlinger 1994b; Korenko and Pekar 2011; Danneels et al. 2013; Martinson et al. 2014; Mrinalini et al. 2014).

Given this background, a review of parasitoid wasps and their venoms is highly relevant and essential for complete representation of venomous organisms in toxinology. This chapter presents to the reader the evolutionary history of parasitoid wasps and their parasitic lifestyle and insights into the evolution of parasitoid venoms and their functions and, finally, notes on recent advances and future directions in parasitoid venom research.

Evolution of Parasitic Wasps

A recent phylogeny using ~1500 protein-coding genes estimates the origin of Hymenopteran stem lineages, including those of parasitoid wasps, in the Late Carboniferous period (Misof et al. 2014). The first parasitoids appeared around 160 mya (Whitfield 1998), and the parasitic lifestyle had a single origin in the common ancestor of sister groups Orussoidea and Apocrita (Whitfield 1992; Pennacchio and Strand 2006; Heraty et al. 2011). Extensive diversification of established Hymenopteran lineages occurred during the Early Cretaceous period (Misof et al. 2014). Present-day parasitoid wasps are highly speciose, representing 10–20% of known insect species and >75% of the ~320,000 known species in Hymenoptera (Whitfield 2003; Pennacchio and Strand 2006). However, social Hymenopterans (e.g., ants, bees, and wasps), which make up 2% of all insects (Holldobler and Wilson 2008), are the more frequent representatives of the venomous Hymenoptera in toxinology.

High levels of species diversity and cryptic morphology have made the resolution of parasitoid wasp evolution and taxonomy exceptionally difficult but also very interesting. The extent of biodiversity in parasitoid Hymenoptera is being realized only in recent years. There are two major Hymenopteran superfamilies consisting mostly of parasitoid wasps: Chalcidoidea and Ichneumonoidea. Chalcidoidea is estimated to contain ~500,000 species with only 23,000 currently described (Noyes 2000; Heraty and Gates 2003; Heraty 2009; Munro et al. 2011; Noyes 2014). Ichneumonoidea is predicted to consist of ~100,000 species (Gauld et al. 2002), although this could likely be an underestimate since a single family Braconidae (Ichneumonoidea) was recently estimated to consist of 46,000 species (Rodriguez et al. 2012).

Given that each parasitoid wasp species synthesizes around 100–150 venom proteins on average (de Graaf et al. 2010; Goecks et al. 2013), cataloging and investigating venoms from all parasitoid species is a monumental task, regardless of venom sharing among lineages. To date, comprehensive knowledge of venom protein repertoires exists for only seven parasitoid species (de Graaf et al. 2010; Vincent et al. 2010; Werren et al. 2010; Zhu et al. 2010; Goecks et al. 2013; Burke and Strand 2014; Colinet et al. 2014a). This barely scratches the surface of the diversity in parasitoid wasp species.

The Parasitic Lifestyle

Parasitoid wasps are holometabolous or metamorphosing insects, and they complete their development on or inside the hosts that they parasitize. Parasitoid species are highly diverse in morphology, life strategies, host preferences, venom systems, and venoms. Parasitoid wasps can be generalists, parasitizing a range of host species spanning diverse orders of Insecta, or they can be specialists, using a single host genus or species. They can be gregarious, laying large clutches of eggs per host, or solitary, laying a single egg per host. Different species specialize on hosts that are at different life stages and can be categorized as egg, larval, pupal, or adult parasitoids.

Parasitoids can be further characterized by the stage at which their hosts are killed. Venom injection by **idiobionts** immediately arrests host development, whereas following venom injection by **koinobionts**, the host continues to develop until the growing parasitoid larvae kill it at a later stage. For instance, some parasitoids such as *Chelonus inanitus* (Braconidae) inject venom and oviposit inside host eggs; however, the adult wasps emerge when hosts are at late larval stages (egg-larval parasitoids). In such cases, different parasitic strategies are used to survive as the host continues to develop (Kaeslin et al. 2005). Koinobionts are typically endoparasitoids and they lay their eggs within the host body cavities or in specific host tissues. In contrast, idiobionts are mostly ectoparasitoids that lay their eggs on the surface of host integument, and their developing young feed by puncturing the host integument. Combined analysis of genetic and morphological data indicates that the basal parasitic clades in Apocrita were ectoparasitic, and the endoparasitic lifestyles were derived later (Dowton and Austin 2001).

Because of the differences in host interactions, venom systems in ectoparasitoids and endoparasitoids are expected to be under different selective pressures. Moreover, generalist idiobionts can also display host-switching behavior, and this can cause rapid adaptations that work toward increasing offspring survival in short durations (Tschopp et al. 2013; Jones et al. 2015). Therefore, venom protein compositions of ectoparasitoids and endoparasitoids can generally be expected to be different. In addition, parasitic venom action is often supplemented by various non-venom secretions and developmental strategies to ensure maximal survival and fitness of parasitoid young. In the case of endoparasitoids, where development inside the host necessitates overriding host immunity, venoms contain mutualistic

polydnaviruses (PDVs) and virus-like particles (VLPs) that play a role in host immune suppression (Strand and Pech 1995; Asgari and Rivers 2011). Host feeding and salivary secretions of ectoparasitic larvae are known to modulate host immunity and metabolism (Periquet et al. 1997; Richards and Edwards 2002; Nakamatsu and Tanaka 2004), whereas endoparasitic larvae secrete teratocytes into the host to manipulate host growth and metabolism (Dahlman et al. 2003; Basio and Kim 2005; Strand 2014). The endoparasitoids use developmental strategies such as polyembryony (where a single egg clonally divides into multiple genetically identical embryos), since protection and nutritional supply are readily available inside the host (Segoli et al. 2010).

These different lifestyles are likely to influence parasitoid venom repertoires and the modes of parasitoid venom action. The implications of parasitic lifestyles on venom evolution and the contrasts and similarities in ecto- and endoparasitic venom systems are discussed in subsequent sections.

Parasitic Wasp Venom System

The Venom Apparatus

Parasitoid wasps display the greatest diversity in venom apparatus structures than in any other group of venomous organisms. Adaptation to different host species and parasitic lifestyles likely drives this extreme diversity. However, the venom apparatus shares a set of common features across species. The venom apparatus is found only in the female wasp. It is located at the posterior, dorsal surface of the wasp, and is usually well protected under chitinous ovipositor plates that are part of the exoskeleton. Internally, it is attached to the vagina at the proximal end of the ovipositor or stinger (de Graaf et al. 2010). This close interaction between the venom apparatus and the female reproductive system complements their functioning, since venom is injected at the time of oviposition (Fig. 2a).

A typical venom apparatus comprises of one or more venom glands (also known as acid glands), a venom reservoir, and a Dufour gland (also known as alkaline gland) (Fig. 2b). Venom glands are highly variable in size and can be elongated, cylindrical, saclike, or branched (Ferrarese et al. 2009). They are lined with glandular columnar epithelial cells containing secretory granules and vesicular structures that secrete venom proteins (Ferrarese et al. 2009; Formesyn et al. 2012). The Dufour gland is also secretory in nature but is thought to perform lubricating functions in most parasitoids (Formesyn et al. 2012). The venom reservoir is a muscular saclike structure that collects and stores venom, but it may also secrete some venom components (Formesyn et al. 2012). Venom synthesis begins in the venom gland when the adult female wasp emerges from its puparium. Venom accumulates in the reservoir, reaching maximum capacity within the first few days after adult female eclosion (Zhang et al. 2013).

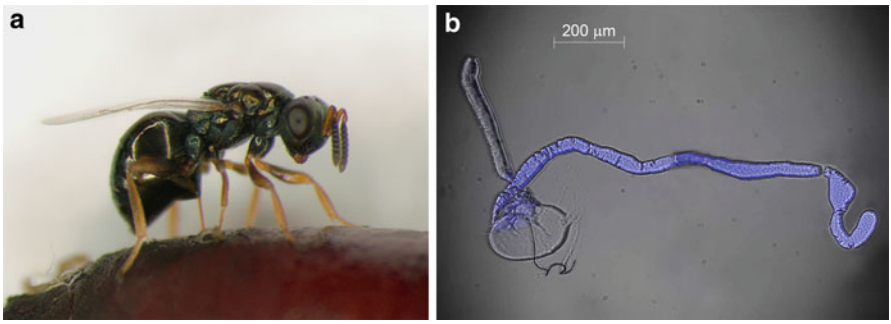

Fig. 2 Venom delivery and venom apparatus in *Nasonia vitripennis*. (**a**) *N. vitripennis* injecting venom into the pupa of its flesh fly host *Sarcophaga bullata*. (**b**) A typical venom apparatus consists of the venom or acid gland that secretes venom, the Dufour or alkaline gland that produces lubricating fluids, and the sac-like venom reservoir that collects and stores venom (Photo courtesy (**a**) Michael Clarke (**b**) Amanda Dolan)

The Ovipositor or Stinger

Venom is delivered into the host by the ovipositor or stinger (Fig. 2a). On a mechanical level, the parasitoid ovipositor is a foldable multitool – it is used as a drill to perforate host structures, a hypodermic needle to inject venom, and a duct to convey and deposit eggs during egg laying. Additionally, when a space separates the adult wasp from the host body, the ovipositor is used to build a feeding tube that allows the wasp to feed on host hemolymph. For example, pupal parasitoids in the genus *Nasonia* build a feeding tube that bridges the space between the puparium and the host integument. When the ovipositor is not in use, it is usually folded back or reeled in and put away.

However, the ovipositor is not just a stinger. It is a highly complex structure that has evolved sensory structures and functions that are adaptations to the wide range of hosts and lifestyles in parasitic Hymenoptera (Quicke et al. 1994; LeRalec et al. 1996; Quicke 1997). The parasitoid ovipositor is comprised of a complex system of valves, ridges, grooves, and stops, which enable steering and orientation during egg laying (Quicke and Fitton 1995; Quicke et al. 1995). *Nasonia vitripennis* uses its ovipositor tip to examine the host puparium and tissues to assess host quality (King and Rafai 1970). It can discriminate between unparasitized and parasitized hosts and the relative time since prior parasitization (Werren 1984). Ovipositor stylets contain different types of sensilla that can be gustatory for discriminating hosts and secretory for lubrication and for thermo-hygroreception (Shah 2012). Endoparasitic *Leptopilina sp.* possess a clip on their ovipositor to grip host larvae and prevent escape until the venom induces paralysis (Lenteren et al. 1998). In the jewel wasp, *Ampulex compressa*, the ovipositor performs mechanosensory functions that help locate and directly inject venom into the brain of its cockroach host (Gal et al. 2014).

Venom Components

Parasitic wasp venom can be a complex mixture of proteins, polydnaviruses (PDVs), virus-like particles (VLPs), microRNAs, small molecules, and ovarian fluids. Given the variety of parasitoid ecologies and host interactions, it is attractive to compare venom compositions among parasitoids with contrasting lifestyles. However, with the limited parasitoid venom analyses currently available, such a synthesis is premature and would likely result in artifacts of incomplete knowledge rather than realistic representations. Therefore, the following sections provide a brief account of venom components in the context of two broad parasitoid categories, ectoparasitoids and endoparasitoids. Specific examples demonstrating host diversity and modes of venom action in the two parasitoid groups are discussed later in the chapter.

Venom Proteins

Parasitic wasps secrete anywhere between 0.04 and 180 ug of venom protein, depending on the species (Parkinson and Weaver 1999; Uçkan et al. 2004). So far, venom proteins in 17 parasitoid wasp species have been examined at some level (Poirie et al. 2014), although comprehensive proteomic analysis has been undertaken in only seven species (de Graaf et al. 2010; Vincent et al. 2010; Werren et al. 2010; Zhu et al. 2010; Goecks et al. 2013; Burke and Strand 2014; Colinet et al. 2014a). A large proportion of these proteins are enzymatic in nature and resemble insect metabolic enzymes (Asgari and Rivers 2011); however, the functions of the vast majority are still unknown (Poirie et al. 2014). Moreover, many parasitoid venom proteins have no homology to proteins in other organisms (de Graaf et al. 2010; Werren et al. 2010; Poirie et al. 2014).

Ectoparasitoid venoms have been studied for years (Rivers and Denlinger 1994a, b, 1995; Coudron and Brandt 1996; Periquet et al. 1997; Rivers et al. 2002, 2006; Nakamatsu and Tanaka 2003; Tian et al. 2010; Ye et al. 2010); however few comprehensive analyses of ectoparasitoid venom protein repertoires have been performed so far. The most complete ectoparasitoid venom data exists for *N. vitripennis*, which contains at least 79 venom proteins (de Graaf et al. 2010; Werren et al. 2010). These venoms have been categorized into eight functional categories: proteases/peptidases, protease inhibitors, esterases, carbohydrate metabolism, DNA metabolism, glutathione metabolism, recognition and binding proteins, and immune-related proteins (de Graaf et al. 2010). Twenty-three venom proteins of *N. vitripennis* do not share homology to any known protein and therefore are of unknown origin and function (de Graaf et al. 2010). The role of specific ectoparasitoid venom proteins in causing developmental arrest, immune suppression, and increased lipid content in the host has been investigated in species from the genus *Euplectrus* (Coudron and Brandt 1996; Nakamatsu and Tanaka 2003) and in *Eulophus pennicornis* (Price et al. 2009).

Among endoparasitoids, however, comprehensive venom protein repertoires of six species have become available in recent years: *Chelonus inanitus* (Vincent et al. 2010), *Microplitis demolitor* (Burke and Strand 2014), *Aphidius ervi* (Colinet

et al. 2014a), *Leptopilina boulardi* and *Leptopilina heterotoma* (Goecks et al. 2013), and *Pteromalus puparum* (Zhu et al. 2010). It is also well established that venoms of some endoparasitoids contain mutualistic polydnaviruses and virus-like particles that are involved in host immune suppression (discussed in detail in the next section). Endoparasitoid venom proteins include neurotoxin-like/paralytic factors, such as pimplin in *Pimpla hypochondriaca*, that induce transient paralysis in contrast to the permanent host paralysis induced by ectoparasitoids (Parkinson et al. 2002; Asgari and Rivers 2011). However, several ectoparasitic venom categories and specific venoms are shared with the more derived endoparasitic wasps (Asgari and Rivers 2011; Poirie et al. 2014). Among the immune-regulating venoms in the ectoparasitoid *N. vitripennis*, serine proteases are the largest group and are also found in endoparasitoids (Asgari et al. 2003; de Graaf et al. 2010; Zhu et al. 2010; Asgari and Rivers 2011). In the endoparasitoid, *Cotesia rubecula*, serine proteases inhibit host defense via downregulation of prophenoloxidase cascades (Asgari et al. 2003; Zhang et al. 2004). On the other hand, *Pimpla hypochondriaca* has evolved an additional three phenol oxidases to mediate host immunity (Parkinson et al. 2001). Poirie et al. (2014) provide a review of specific venom proteins in 2 ectoparasitoid species and 15 endoparasitoid species.

Viruses and Virus-like Particles

Endoparasitic wasps, which lay their eggs within host tissues, are at a high risk of losing their eggs to host immune responses. Insect immune systems attack foreign bodies by encapsulating them in hemocytes and coating them in a thick melanin layer, a process called the encapsulation and melanization reaction. To override this host defense and ensure survival of their offspring, endoparasitic wasps have evolved novel mutualistic relationship with viruses (Strand and Pech 1995; Asgari and Rivers 2011). Polydnaviruses (PDVs) and virus-like particles (VLPs) are injected into the host along with venom proteins to suppress host immunity.

Research into the evolution and function of PDVs and VLPs is relatively recent but has garnered much interest. PDVs are an endogenous and integral part of endoparasitoid genome (Belle et al. 2002) and are produced in the ovarian calyx of the wasp (Wyler and Lanzrein 2003). PDVs can deliver non-viral wasp genes into hosts to perform immune suppressive functions (Webb 1998; Roossinck 2011; Drezen et al. 2014). PDVs became incorporated into certain groups of braconid and ichneumonid genomes in two independent events (Webb 1998), with braconid PDVs or bracoviruses originating ~74 mya (Whitfield 2002). PDVs function synergistically with the venom proteins or overlap with them in function, although the exact mechanisms are still unclear (Asgari and Rivers 2011).

VLPs are produced in actin-lined canals of the venom gland secretory cells (Ferrarese et al. 2009). They suppress host cellular immune responses by reducing the spreading and adhesive properties of host hemocytes and inducing apoptosis (Suzuki and Tanaka 2006; Suzuki et al. 2008; Asgari and Rivers 2011). Mechanisms of host immune suppression can differ between parasitoids adapted to different ecologies (Schlenke et al. 2007). For example, the generalist *drosophilid* parasitoid *Leptopilina heterotoma* produces highly immune-suppressive venom,

whereas the venom of specialist *Leptopilina boulardi* interferes further downstream of the immunity pathways (Schlenke et al. 2007). The role of VLPs and their evolution with respect to host range warrants further investigation in such cases.

Small Molecules

Venoms of parasitic wasps may also contain small molecules. Scoliid wasps secrete peptides such as bradykinins to block the synaptic transmission of nicotinic acetylcholine receptors and induce paralysis (Konno et al. 2002). *Ampulex compressa* produces and injects dopamine (an amine) or a dopamine-like substance directly into the brain and ganglia of its cockroach host to inhibit locomotion and escape behavior (Weisel-Eichler et al. 1999). However, more comprehensive analyses of the small molecules present in parasitoid venoms are yet to be done.

Gene Regulatory Elements

Recent evidence has shown that venom glands produce gene regulatory elements such as microRNAs (miRNAs) that can control expression of venom genes to alter venom protein secretion (Rendon-Anaya et al. 2012; Durban et al. 2013; Vonk et al. 2013). In snakes, venom gland miRNAs are co-opted from regulatory networks of other organs (Vonk et al. 2013), to cause shifts in venom profiles through regulation of gene expression (Durban et al. 2013). miRNA in scorpions is hypothesized to regulate toxin secretion via post-translational control mechanisms and transcript degradation to reduce RNA abundance (Rendon-Anaya et al. 2012). Colinet et al. (2010) suggest that intraspecific venom variation in parasitic wasps may result from differential binding levels of transcription factors to *cis*-regulatory sequences. In the genome of the ectoparasitoid wasp *N. vitripennis*, ~100 miRNAs have been found, although tissue specificity and function of these are yet to be established (Werren et al. 2010).

In contrast, little is known about the role of miRNA themselves as venom components for modulating gene expression in the target envenomated species. This is clearly an interesting topic for future exploration.

How Parasitoid Venoms Function

Venom Functions in Insect Hosts

The earliest parasitoid wasps in Apocrita parasitized larvae of Coleoptera (beetles) (Dowton and Austin 2001). The range of insect host species rapidly expanded along with the evolution and diversification of Insecta. Modern-day parasitoid wasps are known to attack every order of Insecta, and insect hosts are parasitized in many diverse ways.

A substantial number of studies have been aimed at understanding differences in ecto- and endoparasitic wasp interactions with hosts. Insect ecto- and endoparasitoids employ different strategies of host manipulation. Most ectoparasitoids are idiobionts, i.e., they arrest host growth and development.

Endoparasitoids are usually koinobionts – they allow hosts to continue to grow and develop. The following sections provide an overview of mechanisms by which ecto- and endoparasitoids manipulate their hosts.

Ectoparasitic Host Manipulations

Most ectoparasitoids are idiobionts. It is a general perception that ectoparasitoid venom has mainly evolved to cause developmental arrest in hosts and that ectoparasitoids harvest nutrition from hosts in this arrested state. However, ectoparasitic manipulations of the host are more complex and include alteration of metabolism and physiological state of the host, presumably in order to produce a more nutritious environment for the developing young. Venom functions are best characterized in the model ectoparasitoid *N. vitripennis*, which secretes at least 79 venom proteins and parasitizes a range of flesh fly, blowfly, and muscoid fly pupae (Rivers et al. 2006; Werren et al. 2010). *N. vitripennis* venom induces a diapause-like developmental arrest in host pupae (Rivers and Denlinger 1994a). Eye pigment deposition and bristle formation normally seen in developing hosts fail to occur in envenomated hosts. However, rather than completely arresting bodily functions, venom causes targeted changes in physiology and metabolism of the host (Danneels et al. 2013; Martinson et al. 2014; Mrinalini et al. 2014). The set of metabolic symptoms produced by *N. vitripennis* venom emulates some aspects of natural developmental arrest of insects during overwintering and diapause, but there also are distinct differences (Mrinalini et al. 2014). Venom suppresses oxidative respiration, by manipulating glycolytic and Krebs cycle pathways, and amplifies select pathways (Rivers and Denlinger 1994b; Martinson et al. 2014; Mrinalini et al. 2014). Amino acids and sugar derivatives are enriched (Rivers and Denlinger 1994b; Mrinalini et al. 2014). The fat bodies of hosts increase in lipid content (Rivers and Denlinger 1994b, 1995) and could be compensating for evolutionary loss of liposynthetic abilities in parasitoids (Visser and Ellers 2008; Visser et al. 2010, 2012; Ellers et al. 2012). The extent to which these changes increase host nutritive value and enhance parasitoid fitness is still not clear, although it is assumed.

N. vitripennis venom effects developmental arrest by altering developmental pathway signaling and gene expression (Danneels et al. 2013; Martinson et al. 2014). The central nervous system undergoes neuronal apoptosis (Ratcliffe and King 1969), and biosynthesis of neurotransmitters such as L-DOPA and GABA is dysregulated (Mrinalini et al. 2014). Adult wasp structures never develop as chitin biosynthesis, essential for insect metamorphosis, is downregulated (Mrinalini et al. 2014). Some ectoparasitic venoms can also modulate development of a growing host. Species in Eupelmidae modulate hormonal physiology of ecdysis in their lepidopteran hosts (Nakamatsu and Tanaka 2003). Host larvae never molt, but continue to feed and grow, thereby diminishing the risk of parasitoid larval detachment while providing a food source enriched in proteins and lipids (Nakamatsu and Tanaka 2003).

Ectoparasitoid venoms also manipulate both cellular and humoral immunity of the host. Venom suppresses cellular immunity via reduced melanization, phenol

oxidase activity, and programmed cell death/apoptosis of hemocytes (Rivers et al. 2002; Abt and Rivers 2007; Danneels et al. 2013). Humoral immunity of the host is modulated to increase antimicrobial peptide synthesis, possibly preventing bacterial growth and host decay (Martinson et al. 2014). A more complete understanding of ectoparasitoid venom function will emerge from studies using RNAi knockdown of venom genes combined with metabolic analyses and gene expression analyses in the host (Siebert et al. 2015).

Endoparasitic Host Manipulations

Endoparasitoid eggs are laid inside the host. Once the eggs hatch, the larvae may spend their life partially or completely inside the host. Therefore endoparasitoids target host immune response, development, physiology, as well as behavior.

Endoparasitoids regulate host immunity using venom as well as parasitoid larval secretions (Basio and Kim 2005). The foremost function of endoparasitoid venom is to ablate immune responses for immediate protection of eggs. Venom proteins, PDVs, and VLPs injected into the host suppress encapsulation and melanization responses (Asgari and Rivers 2011). Some species lack PDVs and VLPs, and venom alone is sufficient (Er et al. 2011). Immune suppression by venom can occur at the initial stages or further downstream of immune pathway cascades (Schlenke et al. 2007). A comprehensive review of endoparasitoid venom-induced changes to host immunity has been provided by Er et al. (2011) and Asgari and Rivers (2011). A comparative study of endoparasitoid (*Pteromalus puparum*) and ectoparasitoid (*N. vitripennis*) venom has shown that toxicity to host hemocytes has a broader host species range among endoparasitoids (Zhang et al. 2005).

When endoparasitoid eggs hatch and larvae emerge, they continue to feed and grow inside the hosts. This requires parasitoid larvae to mitigate any remaining host immunity that can kill them. Larvae of parasitoids produce teratocytes – cells derived from the embryo that detach and grow into giant cells while inside the host. Teratocytes may inhibit host phenol oxidase activity in host hemolymph and thereby suppress host immunity (Basio and Kim 2005).

Parasitoids may also capitalize on defensive aposematic coloration of hosts. Adult ladybird beetles are toxic, and they advertise their toxicity with brightly colored dorsal surfaces. *Dinocampus coccinellae* is an endoparasitoid of the ladybird beetle. The parasitoid larva emerges from its host, builds a cocoon around host legs while it is still alive, and therefore decreases the chances of predation by remaining under the aposematic host (Maure et al. 2011).

Endoparasitoids adopt different host manipulation strategies based on their clutch size (Harvey 2000). Solitary parasitoids generally suppress development and the behavior of their host. Hosts of gregarious parasitoids (multiple egg layers) continue to grow and increase in size to provide nutrition for a larger brood.

Behavioral Manipulations of Endoparasitoid Hosts

An interesting aspect of endoparasitism is manipulation of host behavior. Larval secretions of endoparasitoids are known to regulate behavior in spider hosts (Eberhard 2010b); however, adult female parasitoids also secrete venom

components that manipulate host behavior. For example, when *Ampulex compressa* injects venom into its cockroach host, the cockroach shows no escape behavior but grooms itself excessively (Weisel-Eichler et al. 1999; Libersat and Gal 2014). Some parasitoids can induce guarding behaviors that protect developing parasitoids. White cabbage butterfly caterpillars protect *Cotesia glomerata* pupa (Braconidae) against predators using their own silk to build cocoons (Harvey et al. 2008). The caterpillar host (*Thyrinteina leucocerae*) reduces mortality in *Glyptapanteles sp*. (Braconidae) by actively defending them against predators using violent head swings (Grosman et al. 2008). *Aphidius nigripes* induces migration of aphid hosts to concealed and protected locations if parasitoid larvae are in diapause (Brodeur and McNeil 1989). It remains to be seen whether these behavioral regulations are induced by venom injection or larval secretions.

Parasitoid Venoms and Arachnid Hosts

Parasitic wasps are known to parasitize ticks and spiders, but the knowledge base on Arachnid host diversity or venom-induced host manipulations is still relatively limited. Ixodiphagus wasps (Encyrtidae) parasitize tick species from several genera and have been proposed as a natural means for controlling tick populations and tick-transmitted diseases (Hu et al. 1998). Spiders are mostly parasitized as eggs and adults, and recently ichneumonid and eupelmid wasps were found to parasitize juvenile ant-eating spiders (Zodariidae) (Korenko et al. 2013). Whether venom can also manipulate development in spiders as in insects is not known.

Spider eggs provide a food source and egg cases provide a protective mantle until the adult wasps emerge. Parasitization of adult spiders is, however, more complex. Adult spiders are exploited as a source of nutrition as well as for their characteristic web-weaving skills (Eberhard 2000). Orb-weaving spiders (Araneidae) normally weave orb webs, but when parasitized by Polysphinctine wasps (Ichneumonidae), they weave "cocoon webs" for the wasp larva to pupate (Eberhard 2000, 2010a; Gonzaga and Sobczak 2011; Korenko et al. 2014). *Neottiura* species (Theridiidae) are used by ichneumonid wasps for their overwintering web-weaving behavior (Korenko and Pekar 2011). Dense webs, originally meant as a survival mechanism against cold temperatures, are instead built around wasp larvae (Korenko and Pekar 2011). Spider species that share the same web type build different types of webs when parasitized by different wasp species (Korenko et al. 2014). In these cases, it appears that venom from adult wasps only serves to temporarily paralyze spider hosts (Eberhard 2010b). The wasp larva, however, controls web-weaving behavior possibly via introduction of neuromodulatory substances into the spider while ingesting its hemolymph (Eberhard 2010b).

Perhaps the most charismatic of Arachnid hunters is the tarantula hawk wasp (Pompilidae). It stings and paralyzes tarantulas (Theraphosidae) and stores them in burrows for its solitary offspring (Cazier and Mortenson 1964). At 3–5 cm in length, it is rather large compared to other parasitic wasps, and it is also relatively

long-lived (Schmidt 2004). Its venom performs the dual functions of paralyzing the tarantula host as well as defense. The tarantula hawk wasp is infamous for its sting. Scoring 4.0 on Schmidt's pain index, its sting is considered the most painful yet to be delivered to humans by an insect (Schmidt 1990). The pain is said to cause such an uncontrolled physical reaction that it is recommended the victim "lie down and scream" to avoid risk of injury (Schmidt 2004). The venom, however, lacks other toxic effects, indicating that these wasps likely evolved solely pain-inducing venom components for defense against vertebrate predators (Schmidt 2004).

Venom Evolution

Parasitoid-host interaction can be an arms race between successful parasitization and host resistance to parasitism. This can drive adaptive evolution of both parasitoid venom proteins and host immunity (Kraaijeveld and Godfray 1997; Fellowes et al. 1998; Keebaugh and Schlenke 2012; Goecks et al. 2013). Venom proteins are known to evolve rapidly in closely related parasitic wasp species with different lifestyles. For example, generalist and specialist congeners produce venoms containing unique as well as shared toxins (Schlenke et al. 2007; Goecks et al. 2013). Moreover, the function of parasitic wasp venom in host manipulation for the benefit of its offspring (as opposed to defense and feeding) brings into consideration several distinct aspects of parasitic lifestyle that likely exert evolutionary pressures on venom toxins. Host-switching behavior in generalist idiobionts, due to relative abundance of hosts, can cause adaptive venom evolution within short durations to increase offspring survival (Tschopp et al. 2013; Jones et al. 2015).

In endoparasitic wasps, clutch size, egg mobility inside the host, and time to larval emergence could drive variations in venom effects on host growth and immunity (Harvey 2000; Schlenke et al. 2007). In particular, host immune responses can result in encapsulation of the parasitoid egg through melanization responses. There is ample evidence for genetic variation in ability to encapsulate the eggs of different wasp species, and this genotype matching could be a driving force for antagonistic coevolution of parasitoid venoms and host immune responses (Kraaijeveld and Godfray 1997; Fellowes et al. 1998). In contrast, because ectoparasitoid eggs are external to the host, they avoid the need to evade host immune responses. Nevertheless, ectoparasitoid venom could evolve rapidly following host shifts and changes from specialist to generalist host use.

Models of Venom Evolution

Key questions in venom biology are: what is the origin of venom genes and how do venoms evolve? The gene birth-death model (Nei and Rooney 2005) is widely invoked to explain the evolution of venom genes. In their review paper, Casewell et al. (2013) provide an excellent scheme of genomic mechanisms for toxin gene

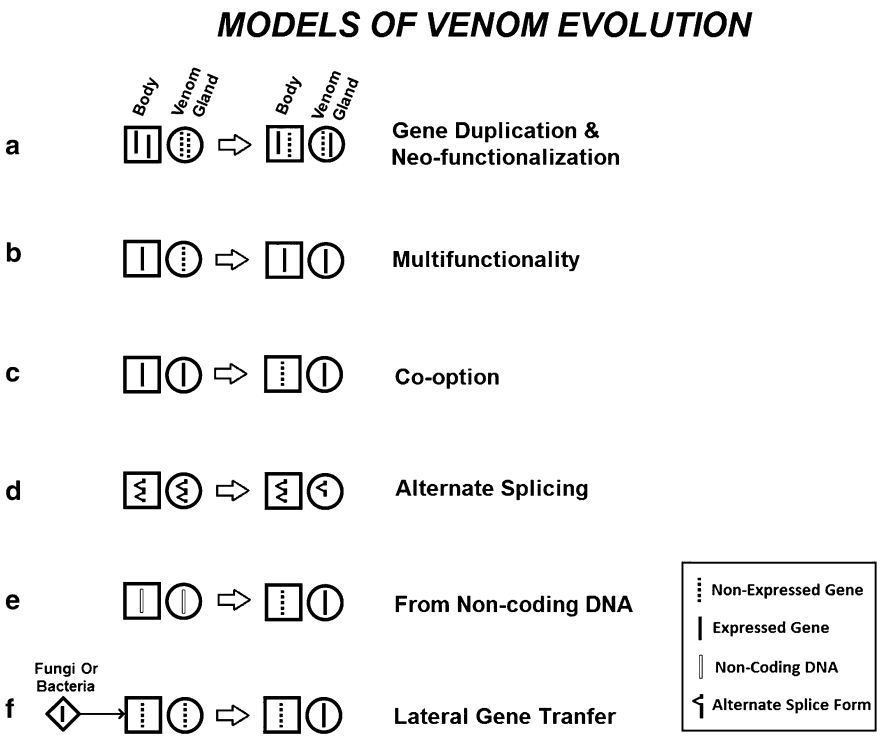

Fig. 3 Models of venom evolution. Schematic representations are provided for six different mechanisms by which venoms can evolve: (**a**) gene duplication and neo-functionalization (Adapted from Casewell et al. 2013), (**b**) multifunctionality, (**c**) co-option of non-venom genes for venom function, (**d**) evolution of alternative splicing of venom transcripts, (**e**) evolution from noncoding DNA, and (**f**) venom gene evolution following lateral gene transfer (LGT) from microorganisms

evolution via gene duplication and recruitment of one paralog as a venom protein through expression in the venom gland. They also discuss gene-level processes, such as toxin domain duplication/loss and alternative splicing (Casewell et al. 2013). However, additional mechanisms are possible. Building on Casewell et al. (2013), the authors here propose six general mechanisms of venom gene evolution (Fig. 3). These are (a) gene duplication and neo-functionalization, (b) multifunctionality, (c) co-option of non-venom genes for venom function, (d) evolution of alternate splicing of venom transcripts, (e) evolution from noncoding DNA, and (f) venom gene evolution following lateral gene transfer (LGT) from microorganisms.

Gene Duplication and Neo-functionalization

The gene birth-death model is a common mechanism for the origin of new genes and evolution of novel functions via duplication of existing genes and divergence of the paralogs (Nei and Rooney 2005) (Fig. 3a). Asgari and Rivers (2011)

propose that parasitoid venoms are likely to have evolved by this mechanism, where normal metabolic insect genes undergo duplication, which is followed by recruitment of a paralog to venom gland expression and its functional divergence. However, a systematic analysis of the parasitoid venom proteomes has not yet been done to determine the origins of venom proteins. Therefore, it remains an open question whether the classic model is the most common means of venom evolution.

Multifunctionality

The second method of venom evolution is multifunctionalization, which can occur via regulation of gene expression (Fig. 3b). In this scenario, a preexisting non-venom protein (e.g., expressed in the whole body or other tissues) is additionally recruited for venom function via expression in the venom gland. For example, enzymatic genes that are normally expressed in the body can acquire toxin functions when they start becoming expressed in the venom glands. Such venom proteins that have both "normal" physiological function and venom function will be constrained in optimization as a venom protein due to the requirement of also maintaining their standard role. Two mechanisms by which this constraint could be removed over evolutionary time are (a) co-option and (b) alternative splicing.

Co-option of Non-venom Genes for Venom Function

Venom evolution via co-option occurs when gene expression becomes restricted to the venom gland, and there is a loss of expression or decreased expression in other parts of the body (Fig. 3c). Co-option can result from disruptive selection induced when the fitness benefit of producing a venom protein is high, but there is a lesser loss of fitness from eliminating expression in other tissue(s). Therefore co-option is only likely when the normal physiological function of the gene is non-vital. The authors thereby hypothesize that co-option may be the most frequent mechanism of venom gene recruitment because it can occur by rapid evolution of *cis* regulation of gene expression. However, demonstrating co-option requires detailed knowledge of expression patterns of newly evolved venom genes in different tissue types and life stages from a set of related species.

Evolution of Alternative Splicing of Venom Transcripts

Disruptive selection on a gene serving both "normal" and venom function can lead to the evolution of alternative splicing in different tissues (Fig. 3d). How quickly alternative splicing can evolve remains an open question, but once it has occurred, venom-specific exons are freed to specialize for toxic function. In addition, post-genomic mechanisms such as post-translational modifications can cause differences in venom protein compositions among species in spite of shared genotypes (Casewell et al. 2013). For example, viperid snake species can regulate venom protein compositions at transcriptional (alternative splicing), translational, and post-translational levels (Casewell et al. 2014).

Evolution from Noncoding DNA

Sometimes, novel protein-coding genes can evolve from noncoding DNA that is not a preexisting gene in the organism (Tautz and Domazet-Loso 2011). New functional genes can be derived from noncoding DNA via acquisition of transcription start sites and gene expression (Fig. 3e). Noncoding DNA could therefore be a potential source of genetic material for evolution of toxin genes that subsequently acquire venom function. However, the frequency with which this occurs among venom genes is unknown.

Venom Gene Evolution Following Lateral Gene Transfer (LGT) from Microorganisms

LGTs are horizontal transfers of genetic material from one organism to another. Lateral gene transfers from prokaryotes and single-celled eukaryotes to metazoans were considered to be rare but are being increasingly discovered in insects (Dunning Hotopp et al. 2007; Dunning Hotopp 2013), including in the ectoparasitoid *N. vitripennis* (Werren et al. 2010). This genetic material can become incorporated into eukaryotic host genomes and acquire novel functions when there is a selective advantage. Therefore prokaryotic LGTs can be a source of genetic material for the evolution and recruitment of genes for venom function (Fig. 3f). Recently, *endochitinase*, a protein synthesized by the venom gland of parasitoid wasps, was found to be horizontally transferred from microsporidia (Martinson et al. 2016). An RNAi knockdown of the gene suggests that it may play a role in dysregulating chitin metabolism of hosts and in defence of hosts against opportunistic fungi (Martinson et al. 2016).

Questions to Explore in Parasitic Venom Evolution

As discussed previously, parasitoids are among the richest group in terms of species diversity, morphology of venom glands, and lifestyles. Adaptations to different hosts and parasitic ecologies likely have implications for venom protein evolution. Among the several questions that arise are: how are new venom proteins recruited and from what sources? What is the origin of parasitoid toxins that have no homology to proteins in other organisms? How does the venom repertoire change when parasitoids switch from a generalist to a specialist host range (or vice versa)? What are the venom compositional differences in ectoparasitoids and endoparasitoids that share the same host? Is there geographic variation in venom within the same species and do individual parasitoids alter their venom profiles in response to host use? How do the repertoires differ between egg, larval, pupal, and adult parasitoids? What venom proteins are conserved across diverse parasitic species, and finally, is there evolutionary convergence of venom components among parasitoids, bees, ants, wasps, arachnids, and other venomous organisms?

The first step toward answering these questions is cataloging the venom repertoires in different species of parasitoids. To date, investigation of parasitic venoms

has mostly focussed on functions of individual toxins, usually of high abundance, whereas relatively complete venom repertoires are known for only seven species (de Graaf et al. 2010; Vincent et al. 2010; Werren et al. 2010; Zhu et al. 2010; Goecks et al. 2013; Burke and Strand 2014; Colinet et al. 2014a). Parasitic wasp venom protein analysis was difficult until recently due to their small size and smaller venom yield. However, high-throughput mass spectrometry-based proteomic analysis has now made de novo sequencing of parasitoid venom proteomes possible. When used in tandem with venom transcriptomes, locus-based identification of venom proteins can be performed. These discovery-based approaches are key to cataloging and understanding parasitic venom evolution and are now an area of active research (de Graaf et al. 2010; Zhu et al. 2010; Colinet et al. 2013; Goecks et al. 2013).

Recent Advances

Technological advances in recent years have made venom analysis in parasitoid wasps possible. Liquid chromatography coupled with mass spectrometry is a high-throughput proteomic method that can obtain protein sequences from a quantity as small as 5–10 ng (Dr. Sheng Zhang, personal communication). Additionally, two new methods of venom functional analysis have recently been used very successfully: metabolomics and RNAi knockdowns. Metabolomics is a recently developed toolset in the "omics" field. Mrinalini et al. (2014) used metabolomic analysis to characterize the changes in 249 biochemicals in the insect host *Sarcophaga bullata* after envenomation by *N. vitripennis*. The data from this study, used in conjunction with pathway analysis, provided a global understanding of the biochemical changes brought about by the venom of this ectoparasitoid (Mrinalini et al. 2014). Atypical regulation of host glucose and sorbitol metabolism was found in this study and provides a basis for exploring applications of *N. vitripennis* venom in diabetes research (Mrinalini et al. 2014).

The second new approach in venom function studies combines RNAi knockdown of venom genes in the venomous species with RNA sequencing in the envenomated target species (Siebert et al. 2015). The RNAi method uses double-stranded RNA to severely reduce (or knock down) the expression of genes of interest and has been successfully developed in the ectoparasitoid *N. vitripennis* (Lynch and Desplan 2006; Werren et al. 2009). After a venom gene of interest is knocked down, the wasp is allowed to sting the host, and venom function is assessed by performing RNA-seq analysis of the host to characterize gene expression changes. When this data is compared to gene expression of hosts injected with whole venom (Martinson et al. 2014), it is possible to subtractively assess the function of the knocked-down venom gene. Therefore, RNAi/RNA-seq method can be used both to test hypotheses concerning functions of particular venom components and as a potent hypothesis generation tool.

One finding of RNAi/RNA-seq studies is that some venom components have compensatory effects, i.e., reducing alteration in the host by total venom of certain

target genes and pathways. Therefore the authors propose that compensatory effects are likely an important function of different components in the venom repertoire. An analogy to the "drug treadmill" can be made. That is, some venom proteins play a role in reducing and modulating negative effects of other venom proteins. In the case of parasitoid venoms, this occurs in order to keep the host alive long enough for the desired alternations in host metabolism to occur.

Finally, the application of parasitic wasp venom for pharmacological purposes is being explored using mammalian cell lines. *N. vitripennis* venom applied to fibrosarcoma cells has been found to suppress NF-kB gene activity that drives deleterious inflammatory responses, tissue damage, and tumor growth (Danneels et al. 2014).

Future Directions

Rapid advancement of parasitic venom research is needed to establish parasitoid wasps in toxinology. Several studies have already established discovery-based analysis as a proof of concept for characterization of parasitoid venom repertoires. While the need of the day is to collect and analyze venom from different lineages of parasitoids, it is important to recognize that the extent to which venom can be characterized is dependent on the technology used. Therefore, it may be essential to draw up guidelines for analysis of parasitic venom. For example, the venom repertoire of a particular species should not be considered completely known unless a locus-based characterization using venom gland transcriptome and/or species genome coupled with venom proteomic analysis has been performed.

Perhaps the greatest challenge in parasitoid wasp toxinology is the sheer number of species and rapid turnover of venom proteins. However, this also presents great opportunities for functional studies and exploring pharmacological uses of parasitoid venoms. Because these insects are tractable for genetic manipulations (e.g., systemic RNAi) and their venoms have evolved specifically to manipulate physiological and metabolic processes in subtle ways, they present an outstanding resource for toxinological research and drug discovery.

Acknowledgments We thank the National University of Singapore, South East Asian Biodiversity Genomics (R-154-000-648-646), and the National Institutes of Health (RO1GM098667) for resources and support.

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Venom as a Component of External Immune Defense in Hymenoptera

11

David Baracchi and Simon Tragust

Abstract

An intriguing feature of most hymenopteran venoms is that they display broad antimicrobial activity. In particular, the venoms of social Hymenoptera (ants, wasps, and bees) represent the most conspicuous source of antimicrobial secretions. In solitary and parasitic species, venom is used to immobilize or kill prey and to preserve them as stored food for their immature brood. In social species, venom is frequently also externalized both onto the cuticle and the nest surface. This indicates that venom use in Hymenoptera is not just restricted to hunting activities or to deter predators, but is also actively used as an externalized defensive agent, providing a first chemical barrier against microorganisms present in the environment. This chapter will discuss the importance and biological significance of venom as part of an external immune defense in Hymenoptera with special emphasis on social species. In addition ecological and environmental factors constraining the use of venom as external immune defense will be highlighted.

Keywords

Antimicrobial peptides • Social insects • Ecological immunology • Social immunity

D. Baracchi (✉)
Research Centre for Psychology, School of Biological and Chemical Sciences, Queen Mary University of London, London, UK
e-mail: david.baracchi@gmail.com; d.baracchi@qmul.ac.uk

S. Tragust
Animal Population Ecology, Animal Ecology I, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, Bayreuth, Germany
e-mail: simon.tragust@uni-bayreuth.de

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_3

Contents

Introduction

A variety of venom systems have evolved across the animal kingdom. This taxonomic diversity highlights the importance of venom as an evolutionary innovation (Casewell et al. 2013). Unsurprisingly, many studies have been conducted to understand the evolutionary processes that drove the generation of these venomous systems and of venom complexity. From this wealth of data, the insight emerged that the complex composition and targeting of venom reflects the multiple functions and biological roles venom has in different animals. From an evolutionary perspective, venoms are commonly regarded as either foraging adaptations to subdue prey or as defensive adaptations against predators (Casewell et al. 2013). Venoms found in the insect order Hymenoptera are certainly not an exception from this point of view (Piek 1986). As in other venomous animals, the composition and function of venom in Hymenoptera are well adapted to immobilize or kill prey, and in many other cases, it serves as a defensive adaptation against enemies such as invertebrate and vertebrate predators. Defense is often also a common secondary function of venom in many species in which foraging is its primary purpose. This conception has led to neglect the fundamental role that venoms play in the interactions with pathogenic, parasitic, commensal, or mutualistic microorganisms. Yet, these microorganisms certainly also represent a strong selective pressure for the maintenance of venom for defensive purposes (Moreau 2013). Indeed, a characteristic of venomous secretions in Hymenoptera is the strong antimicrobial activity that they exert (Kuhn-Nentwig 2003; Moreau 2013). Although this characteristic of venom is broadly distributed among distant hymenopteran species, it has so far been considered to be only of secondary importance. Only recently it became clear that many hymenopteran species, whatever their life styles, have evolved venom features that actively participate in the regulation of microbial infections. This view has come from the recognition that many insects deploy antimicrobials to their immediate environment in order to manipulate the composition of the microbial community surrounding

them. These antimicrobials often originate from exocrine glands, especially from venom glands (Otti et al. 2014).

In this chapter the importance and biological significance of venom as part of an external immune defense in Hymenoptera will be highlighted with special emphasis on those species characterized by social habits. Venom of vertebrates and invertebrates is thought to be metabolically costly and the energetic cost of venom might constrain both its synthesis and use (Casewell et al. 2013; Nisani et al. 2012; but see Smith et al. 2014). Despite that, most social hymenopterans use considerable quantities of venom to sanitize themselves, related group members, and the nest surface, implying that the advantages overcome the metabolic cost.

Immune Defenses in Solitary and Social Hymenoptera

Like all animals, Hymenoptera enlist a variety of immune defenses against disease agents (Schmid-Hempel 2011). From a molecular perspective, the insect immune system involves three core signal transduction pathways, two of which are regulated by pattern recognition receptors (Toll and Imd) and the third one by stress signals from tissues (JAK/STAT). These pathways orchestrate a huge number of molecular effectors, including antimicrobial peptides, reactive oxygen species, and lectins. The system, however, also involves physical barriers to infection such as the integument and the gut. Furthermore coordinated responses of several subpopulations of hemocytes are activated in the hemolymph when these barriers are breached by a putative pathogen.

Apart from these internally expressed immune defenses, there are several other defense mechanisms existing outside of what is traditionally considered to be part of the immune system. Those mechanisms involve, for example, changes in life-history traits (Michalakis 2009) or behavioral avoidance and self-medication (de Roode and Lefèvre 2012; Moore 2002) and clearly contribute to an organism's defense against parasites and pathogens. Social insects also benefit from the fact that they show cooperative defenses that complement the defense of the individual. Thus, insects living in a society can rely on both individual and collective defenses, with selection for immunity acting simultaneously on both these levels, encompassing complex interactions and different selective constraints. One of the most illustrative examples of cooperative defense is the social fever exhibited by honeybees, where an increase of comb temperature is induced by adults in response to infestation by the fungal pathogen *Ascosphaera apis*, preventing disease development (Starks et al. 2000). Other defenses in insect societies include organizational properties of the colony that are critical in shielding infectious diseases (Schmid-Hempel 1998; Stroeymeyt et al. 2014). For example, in the colonies of ants and bees, the inner region of the nest containing immature brood, young workers, and the queen is spatially and behaviorally segregated from older workers, which are mainly active outside the nest foraging or in the nest periphery disposing of dead bodies and garbage (Baracchi and Cini 2014; Mersch et al. 2013). The spatial segregation emerging from division of labor and preferential age and task-based interaction leads to a form of

organizational immunity protecting the more important and delicate region of the nest from possible infections.

Besides indirect effects of behaviors through organizational immunity, behaviors can have a more direct effect on immune defense. Behaviors targeted at decreasing disease transmission and increasing resistance to parasites and pathogens within a social insect colony have been referred to as antiseptic behaviors (Wilson-Rich et al. 2009). Antiseptic behaviors include a large repertoire ranging from the hygienic removal and undertaking of diseased brood and young adults in ants and bees (Baracchi et al. 2012a; Sun and Zhou 2013; Tragust et al. 2013a, b) to mutual grooming behavior (Evans and Spivak 2010; Tragust et al. 2013a).

The use of antimicrobials against parasites and diseases in insect societies is intimately linked to behavioral adaptations as they are required to apply and distribute antimicrobial compounds as a first line of defense. Antimicrobials acting in the environment of a social insect colony might be environment derived, derived from symbiotic relations, or self-produced.

Ants and bees often disinfect their nest material with resins, i.e., complex plant secretions with diverse antimicrobial properties, derived from the environment. In the wood ant, *Formica paralugubris*, resins have been shown to inhibit the growth of microbes, and nests rich in resins have fewer bacteria and fungi than ant nests containing only very little resin (Christe et al. 2003). Even if resin collection might be costly in term of time and effort, there are indications that wood ants benefit directly from the antimicrobial property of resin as they survive longer if infected by bacteria or fungi (Chapuisat et al. 2007). Similar behaviors are also common in the honeybee, *Apis mellifera*, and other honeybee species where resins are actively included into the wax of the nest to form what has been called propolis. This behavior is clearly an adaption to fight pathogens, as colonies of *Apis mellifera* increase resin foraging rate after a challenge with the fungal pathogen *Ascophera apis*. Additionally, colonies experimentally enriched with resin had decreased infection intensities of this fungal pathogen (reviewed in Simone et al. 2009).

In addition to antimicrobial active plant resins, the antimicrobial immune defense of social insects also relies on antimicrobials gained through symbiotic relationships. It has recently been shown that members of all nine recognized honeybee species, plus stingless bee species, harbor diverse symbiotic lactic acid bacteria that are involved in food preservation. In addition those symbiotic bacteria likely also contribute to host defense against pathogens and parasites intercepted during foraging (Vásquez et al. 2012).

Besides antimicrobial compounds derived from the environment and from symbionts, social insects produce a variety of antimicrobial secretions in their exocrine glands, especially ants, and use them to sanitize their own body and their nest. Until recently, the role of venom as a major source of self-produced antimicrobial compounds has often been neglected, despite the fact that most venoms show a strong antimicrobial activity (Kuhn-Nentwig 2003).

Altogether, organizational, behavioral, and physiological adaptations of social insects to prevent the establishment and spread of parasites and pathogens have been referred to as social immunity (Cremer et al. 2007). The key idea is that by acting

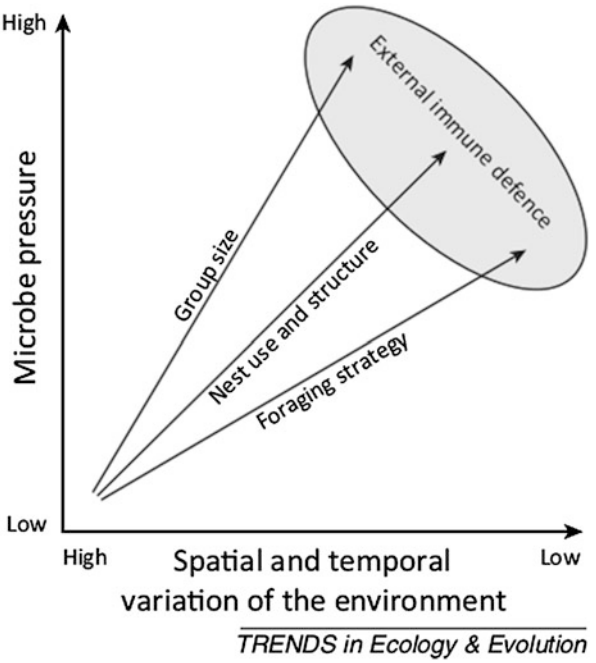

Fig. 1 Selection for external immune defense. Three gradients of important ecological factors, in combination with microbe pressure and spatial or temporal variation in the environment, favor the evolution of external immune defenses. Selection pressure will increase (i) from small to large group size, (ii) from temporary/open to permanent/confined nests, and (iii) from no food storage/slow decay to permanent food storage/fast decay (Reprinted from Otti et al. (2014) with permission of Cell Press)

collectively, individuals are better able to mount a defense than is possible acting independently. The idea of a social immune system has been later expanded to include immune services targeting one or more recipients not only in social insects but also in other animal family structures, in social microbes, or in the context of herd immunity, i.e., the reduction of the risk of infection among susceptible individuals by the presence and proximity of immune individuals (Cotter and Kilner 2010). With the focus on immune defense of organisms in general, it was recently proposed to view any heritable trait acting outside an organism and improving the protection from pathogens or manipulating the composition of the microbial community in favor of an organism, as external immune defense (Otti et al. 2014). This broad definition of immune defense integrates ideas on social immunity and proposes that the expression of internal or external immune defenses will depend on the ecological niche or life history of an organism. Furthermore it provides a framework in which costs and benefits of immune defense traits can be evaluated from an evolutionary and ecological perspective. In particular the framework proposes that variation in the level of microbe pressure present in a given environment and the temporal or spatial variation of the environment itself represent the two most important factors in the evolution of external immune defense and its effectiveness (Otti et al. 2014), (Fig. 1).

Focusing on antimicrobially active venoms, the following sections of this chapter will explore whether the evolution of external immune defense has indeed been favored due to life-history traits found in solitary and social Hymenoptera, i.e., the

storage of food, the use of a stable and confined nest, and group living. However, first, the antimicrobially active venom of Hymenoptera and its biological role and function as external immune defense will be described.

Hymenoptera Venoms: A Complex Multifunctional Secretion

The majority of Hymenoptera have a venom gland associated with the ovipositor or the sting (Piek 1986), (Fig. 2). Details on the function and composition of the secretions of these glands are known for only a part of the over 150,000 hymenopteran species, and for the sawflies (Symphyta) such knowledge is almost completely lacking. Hymenoptera venom glands produce extremely complex cocktails of diverse bioactive compounds. It is possible to distinguish at least three different groups of chemical substances according to their molecular weight (Kuhn-Nentwig 2003; Piek 1986). The first group of heavy compounds (higher than 10 kDa) consists of proteins, including several enzymes such as phospholipases (responsible for cleaving the membrane phospholipids), hyaluronidases (which degrade the matrix component hyaluronic acid), acid phosphatases (acting on organic phosphates), and sphingomyelinases (involved in sphingolipid metabolism reactions). The second group of intermediate molecular weight (around and lower than 10 kDa) is represented by a peptide fraction, including several cytolytic and neurotoxic compounds. A third group is composed of low-molecular-mass substances such as ions, free amino acids, biogenic amines (commonly histamine, serotonin, dopamine, and noradrenaline), neurotransmitters, polyamines, heterocyclic compounds, and alkaloids. Understanding why venoms are such complex mixtures of compounds requires a clear understanding of what is the evolutionary history of venom and what functions it holds in living species.

The Evolutionary History of Venom in Hymenoptera

Traditionally, the order of Hymenoptera has been taxonomically partitioned into two major groups: the Symphyta or sawflies, most of which are phytophagous, and the Apocrita, most of which are entomophagous. The Apocrita can be further divided into the Terebrantia and Aculeata that share common parasitic ancestral origins. Terebrantia have an ancestral ovipositor (terebra or drill) that is also used as venom duct, while Aculeata have an ovipositor (aculeus or sting) that is fully modified for injecting venom into a host and has lost its association with the reproductive system. Terebrantia use their stinging organ to transiently or permanently immobilize prey for their developing offspring and to deposit their eggs inside (endoparasitoids) or outside (ectoparasitoids) the prey's body. In many solitary aculeate species, venom compounds retained their nonlethal paralytic function for the storage and capture of prey while acquiring a new one for use in self-defense (Hermann and Blum 1981). In the social Hymenoptera Aculeata, the venom, originally used as a tool for capturing and storing prey in solitary species, essentially became a weapon for defending the colony from predators and competitors. In addition to serve as injectable or topically

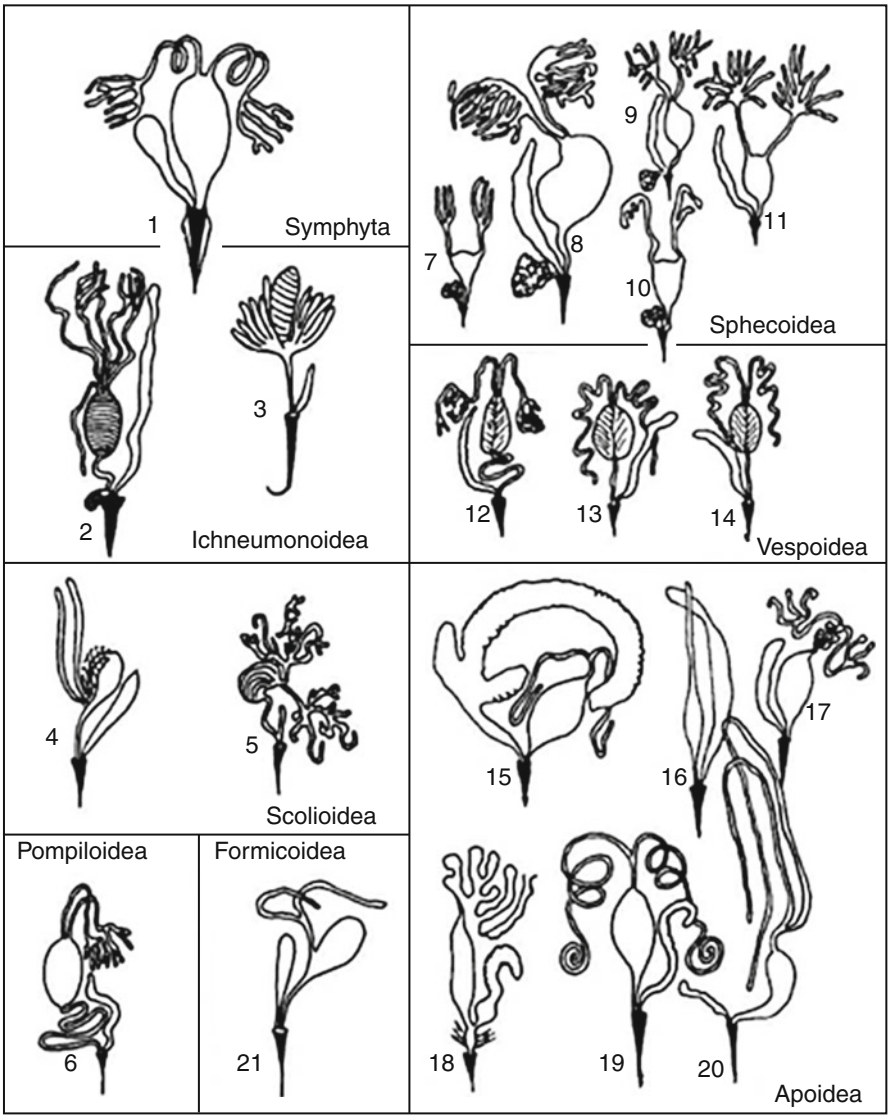

Fig. 2 A selection of types of glandular venom apparatus in Hymenoptera. All representatives show a venom gland, mostly paired and highly branched, and a venom reservoir. The venom reservoir is part of the ductus venatus, except in Braconidae (3). Nearly all show a second gland, the Dufour's gland, which is smaller, unpaired, and not branched, except in some Apoidea (15, 16). In the Sphecidae, a third gland is frequently present (7–10). In some groups the venom bladder is muscular 2, 3, 4, 12, 13, 14 (Reprinted from Piek (1986) with permission of Academic Press)

applied defensive agent, ant venoms are used also as trail, alarm, sex, queen-recognition, aggregation, attractant-recruitment, and recognition pheromones, as repellents, and even as toxic agents for prey capture (Piek 1986).

Venom Use in Solitary and Parasitic Hymenoptera

Besides the well-studied venomous functions of prey capture and defense, the antimicrobial properties of hymenopteran venoms have often been considered of secondary importance although they constitute a function broadly distributed among distant hymenopteran species (Moreau 2013). A hypothesis that could explain the antimicrobial activity in hymenopteran venom is that it serves to prevent the contamination of the venom apparatus by opportunistic pathogens, contracted at the occasion of stinging events. Data in support of this hypothesis are however completely lacking except for a recent survey of bacteria, fungi, and viruses associated with the venom apparatus of Hymenoptera. This survey revealed that the venom apparatus of Hymenoptera is a suitable organ for the development of viruses only and not for other microbes (Moreau 2013). An alternative hypothesis to explain the adaptive significance of antimicrobial venom in solitary and parasitic Hymenoptera is its use to control infection by opportunistic pathogens in stung prey. This makes intuitive sense, especially for parasitoid and solitary species, which need to keep the paralyzed prey alive or from decomposing during the development of their offspring. Furthermore, protection of stored food has been outlined as a likely selective pressure for the evolution of external immune defense traits such as antimicrobially active venom (Otti et al. 2014). Indeed, evidence points to the fact that Hymenoptera, especially parasitoids, appear to have evolved venom-based strategies that limit the opportunity for microorganisms to establish a secondary infection in their host (reviewed in Asgari and Rivers 2011; Moreau 2013). These include the injection of venom antimicrobial proteins and peptides, but also the selective manipulation of the host's immune reactions to the benefit of the parasitoid's offspring. For example, the venom components of the endoparasitic hymenopteran *Leptopilina boulardi* specifically target their dipteran host's encapsulation and melanization responses, but parasitized hosts keep their ability to produce antibacterial and antifungal peptides (Moreau 2013). Another example is the venom of the jewel wasp *Ampulex compressa*, which induces excessive grooming behavior in the stung prey (Libersat and Gal 2014). Both venom-based strategies presumably function to counteract the increased risk of infection, resulting from a complete suppression of the host's immune responses in the case of *Leptopilina boulardi* or from pathogens on the host's cuticle in the case of *Ampulex compressa*, which may be harmful for the wasp's egg or developing larva. Similar to parasitic Hymenoptera, several antimicrobial peptides in the venoms of solitary predatory Hymenoptera are known (Moreau 2013). Although the potential to regulate infections in animals they sting can be envisaged, the exact biological roles are still unclear.

Taken together, the venom in many solitary and parasitoid hymenopteran species holds functions as external immune defense in addition to that of paralyzing hosts. The following sections will show that the antimicrobial activity of venom has been retained in social Hymenoptera and that venom has a biological function as external immune defense also in social species.

Rise of Sociality and the Threat of Predators and Pathogens

In the escalation of parental care, species in which the females of parasitoid Hymenoptera lay their eggs on paralyzed prey, to species in which a solitary female builds a shelter before capturing a prey on which to lay an egg, and then to species in which the growing larvae are kept in a nest and progressively furnished with prey in social Hymenoptera can be found. The nest provides social insects with an element of control over the environment, improving colony capacities for rearing the immature brood through storage of food reserves. Apart from cooperative brood care, living in a society has many other benefits. The fitness of each individual in a group is thought to increase by decreasing the costs associated with important life-history activities such as foraging efficiency, colonizing and competitive abilities, and the ability to adaptively modify the environment. In turn, the social lifestyle requires highly developed defense abilities. The amount of resources offered by insect colonies is likely not only to attract a wide array of potential predators, notably mammals, birds, and various other arthropods, but also several microorganisms to take advantage of it. The high number of, often closely related, individuals living in high densities with frequent physical contact and the shared use of space is predicted to significantly increase the vulnerability of societies to the establishment and spread of infectious diseases. This hypothesis is generally supported by the observation across many different species that the prevalence of pathogens and parasites increases with the size of host social groups (Côté and Poulin 1995; Rifkin et al. 2012) and that numerous parasites and pathogens exist in social insect societies (Schmid-Hempel 1998).

Venom as Externalized Immune Defense in Social Hymenoptera

Several antimicrobial compounds acting against a wide range of bacteria and fungi have been described in the venom of eusocial bees, bumblebees, social wasps, hornets, and ants. The presence of a range of antimicrobial peptides which are used also for internal immune defense is notable. For example, the venom of the honeybee *Apis mellifera* contains melittin, a basic 26-amino acid peptide that accounts for 45–50% of the venom dry weight and exhibits strong antimicrobial activity. Similarly, several antimicrobial peptides named mastoparans have been described in social wasp genera such as *Agelaia*, *Vespula*, *Protonectarina*, *Protopolybia*, *Parapolybia*, *Polybia*, and *Polistes* (Kuhn-Nentwig 2003; Moreau 2013). In ants, the metapleural glands have long been considered to be one of the most important sources of antimicrobial compounds, active against a wide range of bacteria and fungi (Yek et al. 2013). Nonetheless, several antimicrobial peptides have been described also in the venoms of ants, for example, in the Australian jumper ant *Myrmecia pilosula* and in the ponerine ant *Pachycondyla goeldii*. Furthermore, other venom compounds with strong antimicrobial activity (e.g. alkaloids or formic acid (Morgan 2008)) are known from ants, such as the

fire ant *Solenopsis invicta* (Storey et al. 1991) or species belonging to the ant subfamily Formicinae (Tragust et al. 2013a).

Venom on the Cuticle

Interestingly, venom components can be found on the cuticle of social bees, wasps, and ants. The primary function of the epicuticle, the most external layer of the insect cuticle, and the complex mixtures of lipids on it, is to protect against dehydration and to provide a mechanical barrier against invasion of foreign matter. The presence of venom compounds with strong antimicrobial activity on insect surfaces suggests that the venom acts also as a chemical barrier providing a first line of protection against microorganisms. Besides *Polistes* paper wasps (Turillazzi 2006; Turillazzi et al. 2006), the presence of venom components with strong antimicrobial activity on the epicuticle has been recently documented in Stenogastrinae wasps (Baracchi et al. 2010, 2012b). Stenogastrinae wasps are a subfamily of tropical facultative eusocial wasps, closely related to Polistinae and Vespinae, forming simple societies that are very small in size. The medium-molecular-weight polar substances found on the wasp epicuticle (roughly from 900 to 4000 Da) were identical to those found in the venom of all the ten studied species from four different genera, suggesting the venom reservoir as the primary source of cuticular polar substances. Support for the idea that the venom reservoir is the source of antimicrobial compounds on the cuticle comes also from the study of different social bees of the genus *Apis* (Baracchi et al. 2011; Baracchi and Turillazzi 2010). While venom peptides are present on the cuticle of females, irrespective of their colony duties, they can be found only in traces on the cuticle of drones, which lack the sting apparatus (Fig. 3). The fact that newly emerged bees lack venom antimicrobial peptides, both in the venom reservoir and on the cuticle, further confirms this hypothesis. The presence of antimicrobial venom components on the cuticle of ants is known only for the fire ant *Solenopsis invicta*. In this ant species, small quantities of venom are dispensed on the brood surface during a behavior called "gaster flagging" (Obin and Vander Meer 1985) (Fig. 4), and venom components are also deposited on eggs by queens during the egg-laying process (Vander Meer and Morel 1995) (Fig. 5).

The behavioral mechanisms responsible for the presence of venom compounds on the cuticle of bees and wasps are still not completely clear. The most likely explanation is the use of cleaning movements during grooming to smear venom on the body. Self-grooming observations in Stenogastrinae wasps suggest the possibility that little drops of venom released from the sting can be collected with the legs by the wasps and applied all over the body surface (Baracchi et al. 2012b). The importance of grooming for the spread of antimicrobial active substances derived from the venom gland has recently also been shown in the ant *Lasius neglectus* (Tragust et al. 2013a). In this species, adults continuously apply antimicrobial venom onto their pupae. While direct spraying of their venom onto the pupae can be occasionally observed, the predominant mode of application is indirect. Venom is first taken up orally during a behavior called "acidopore grooming" and subsequently applied to pupae during grooming.

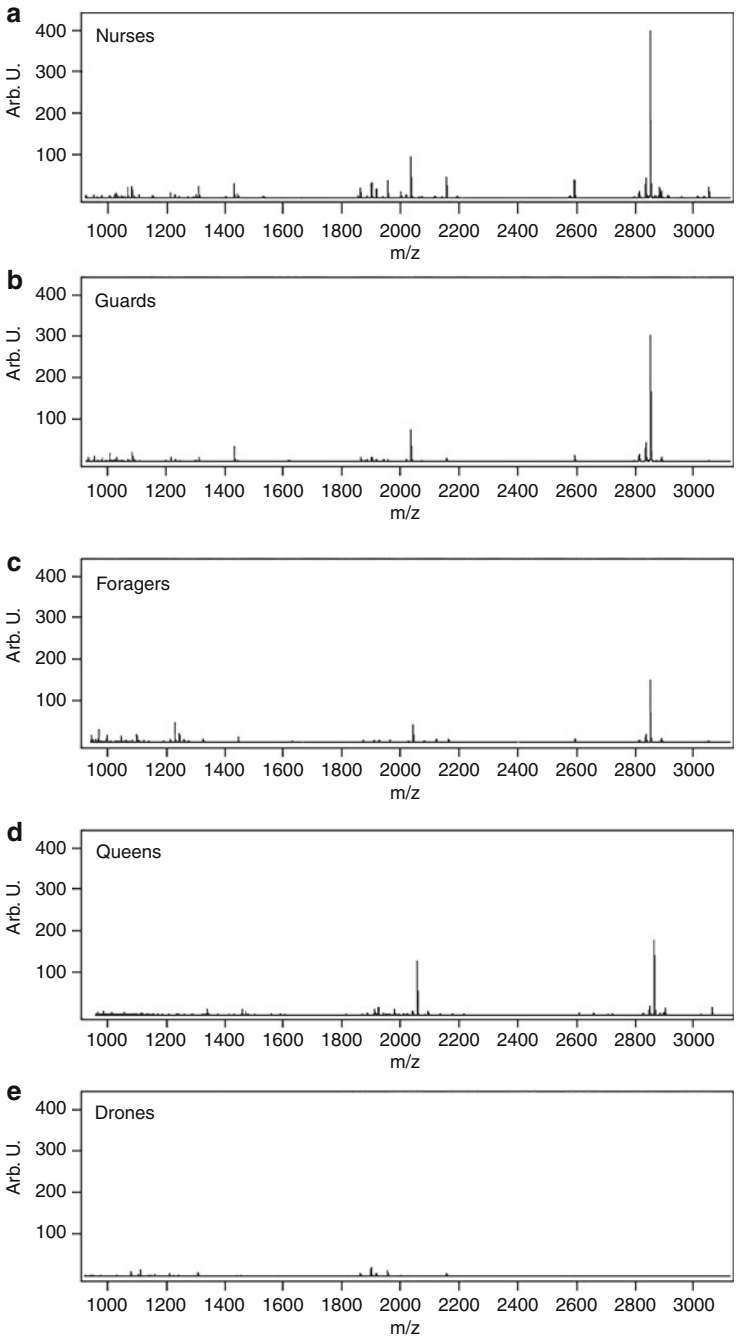

Fig. 3 Average mass spectrometry spectra of 950–4000 Da fraction of cuticular methanol extracts of individuals belonging to different sexes and castes of honeybee (*Apis mellifera*). The highest

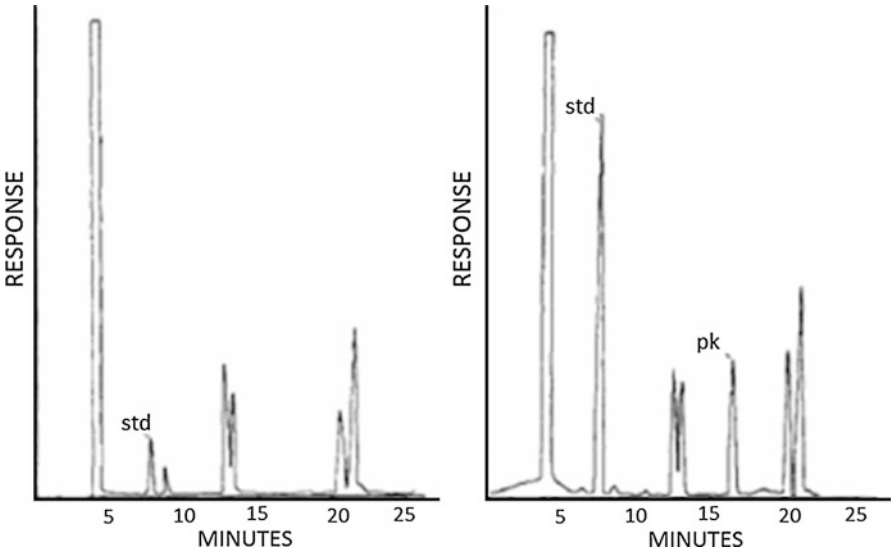

Fig. 4 Gas chromatogram demonstrating the presence of worker-derived venom alkaloids on the surface of *S. invicta* brood. (**a**) *S. invicta* venom alkaloids from dissected worker poison sac (**b**) *S. invicta* brood rise. *Std* internal standard, *un. pk.* unidentified peak (Reprinted from Vander Meer and Morel (1995) with permission of Springer)

Although it is likely that antimicrobial venom components on the cuticle of adults and brood of social bees, wasps, and ants serve as a protection against microorganisms, direct evidence for this hypothesis exists only for ants. Blockage of the venom gland opening in the weaver ants *Polyrhachis dives*, in the fungus-growing ant *Acromyrmex echinatior*, and in the garden ant *Lasius neglectus* all resulted in a reduced survival of adults and pupae cared by them when challenged with the entomopathogen *Metarhizium anisopliae* (Graystock and Hughes 2011; Tragust et al. 2013a; Tranter et al. 2014) (Fig. 6).

In the ant *Lasius neglectus*, the authors could show that formic acid from the venom gland is the active agent inhibiting fungal growth and that venom-depleted ants had a significantly reduced ability to resist such growth (Fig. 7). These authors could also show that application of venom on pupae is amplified under pathogen pressure, indicating that it is an adaptive behavior.

Although, so far, brood care in the ant *Lasius neglectus* is the only example of therapeutic use of the venom in response to pathogens reported in all Hymenoptera, it is likely that future work will reveal that other species of social insects are also

Fig. 3 (continued) peaks at ~2000 Da (apamin) and ~2850 Da (melittin) of each spectrum accounts for ~45–50 % and ~2 % of the venom dry weight, respectively, but only melittin has proven antimicrobial activity (Baracchi et al. 2013) (Reprinted from Baracchi and Turillazzi (2010) with permission of Elsevier)

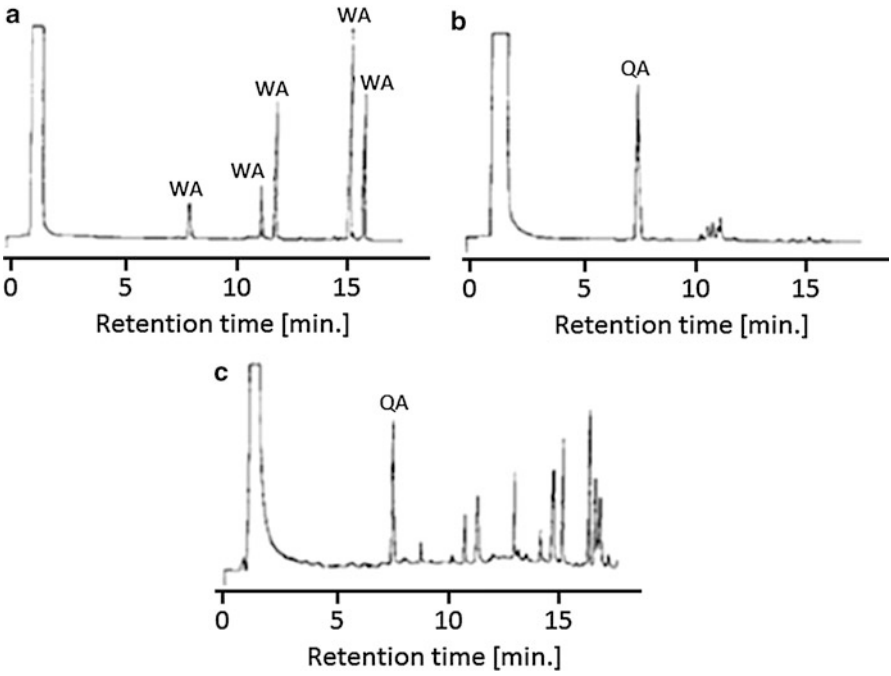

Fig. 5 Comparison of venom alkaloid gas chromatogram profiles: (**a**) worker, (**b**) queen, (**c**) hexane rinse of eggs. *QA* queen-specific piperidine alkaloid, *WA* worker-specific alkaloids. Chromatograms (**a**, **b**) are from worker and queen venom sac extracts, respectively, and are very concentrated compared to chromatogram (**c**) (Reprinted from Vander Meer and Morel (1995) with permission of Springer)

capable to therapeutically defend themselves and related group members from a wide array of pathogens using their antimicrobial secretions.

Venom on the Nest Surface

Venom components are found not only on the cuticle of social bees, wasps, and ants but also on the nest surface, likely also serving as a first-line chemical barrier against microorganisms. For example, the antimicrobial peptide melittin has been described from the nest surface of several species of the genus *Apis* (Baracchi et al. 2011; Baracchi and Turillazzi 2010), and the antimicrobial mastoparan peptides Dominulin A and Dominulin B have been described from the nest surface of the social paper wasp *Polistes dominula* (Turillazzi et al. 2006). In ants, there is only indirect evidence that antimicrobial active venom compounds are found on the nest surface; for example, greater fungal abundance but lower fungal species richness and diversity were detected in mounds of the fire ant *Solenopsis invicta* and in *Aphaenogaster texana* nests (Zettler et al. 2002). An involvement of venom compounds in the

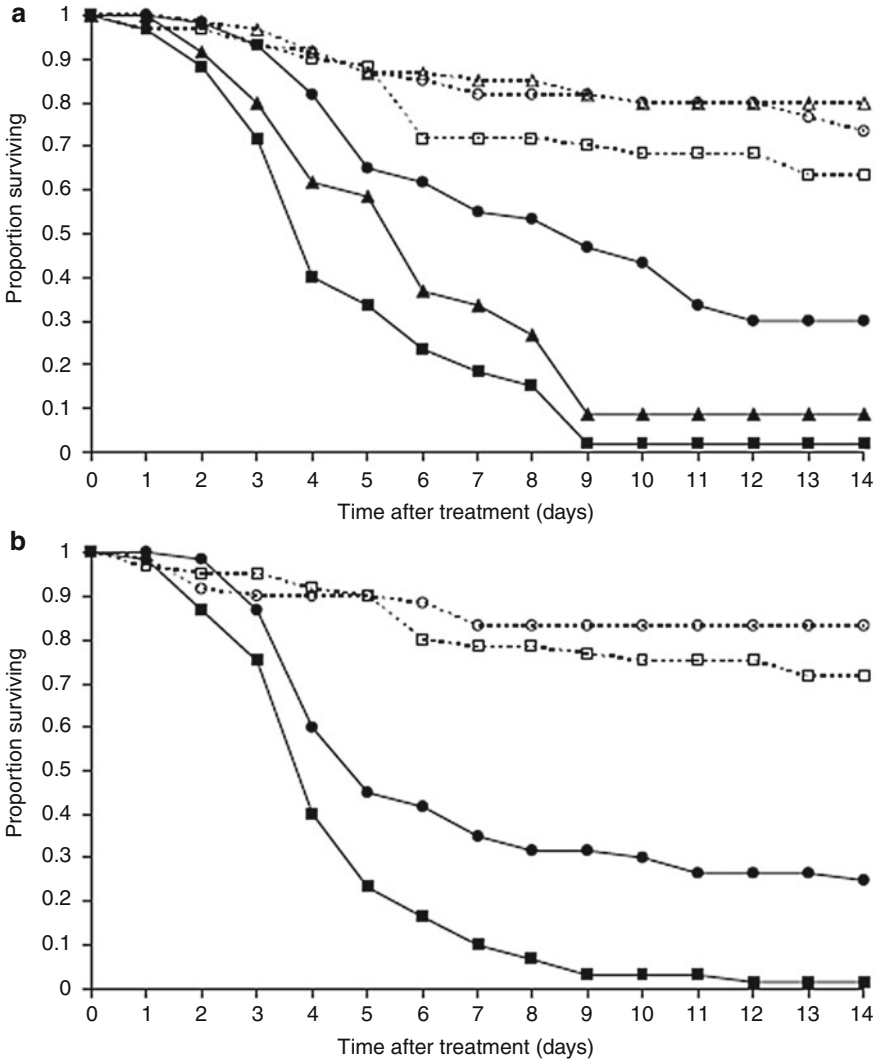

Fig. 6 Survival of *Acromyrmex echinatior* leaf-cutting ants (**a**) and *Polyrhachis dives* weaver ants (**b**) that had either their venom gland (*squares*) or metapleural gland (*triangles*; *A. echinatior* only as *P. dives* lacks a metapleural gland) blocked with nail varnish or had nail varnish applied to the pronotum as a control (*circles*) and which were then treated with either the *Metarhizium anisopliae* fungal parasite (*solid lines*, *filled symbols*) or with 0.05 % Triton-X control solution (*dashed lines*, *open symbols*) (Reprinted from Graystock and Hughes (2011) with permission of Springer)

sanitation of nests is likely for the weaver ant *Polyrhachis dives*. In this species, the blockage of the venom gland opening resulted in an increased risk of the nest material being overgrown by fungi, compared with nest material that was tended by workers with a functional gland (Tranter et al. 2014) (Fig. 8).

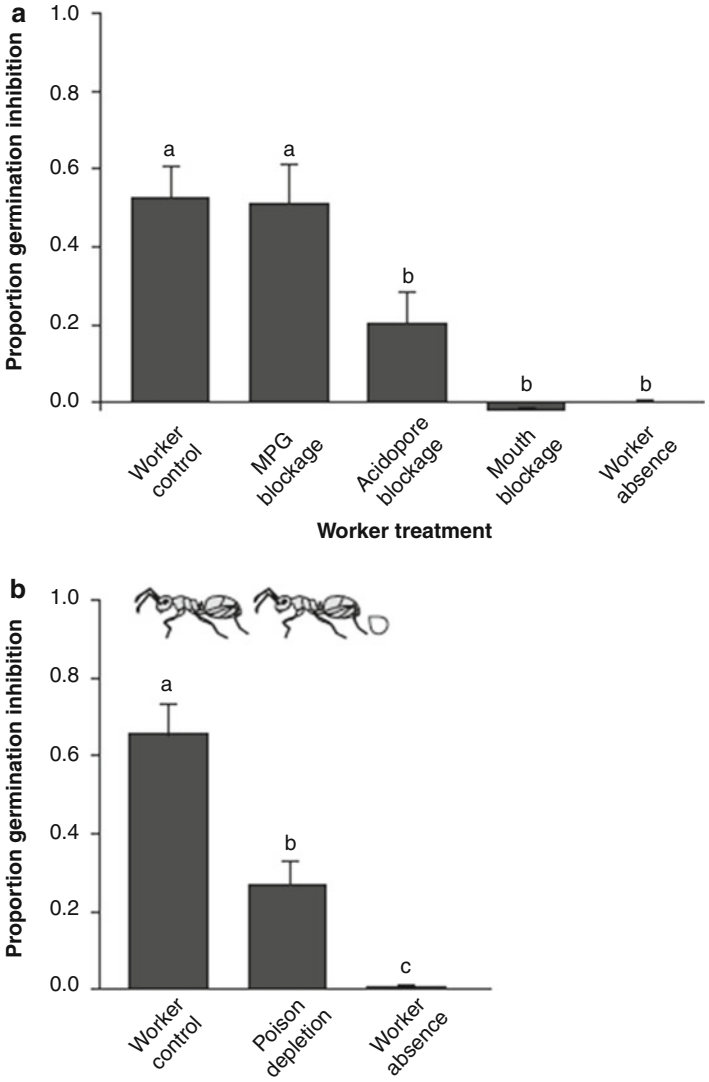

Fig. 7 (**a**) Workers of *Lasius neglectus* inhibited germination of conidiospores on the surface of pupae, as revealed by germination checks of conidiospores washed off after 24 h of tending and subsequently plated on agar. MPG-blocked workers inhibited fungal growth to the same extent as control workers. In contrast, blockage of the acidopore and the mouth prevented this antifungal effect. (**b**) Venom-depleted ants also had a significantly reduced ability to inhibit fungal growth in comparison to control workers, but they still showed some antifungal effect compared to the worker-absence control. *Bars* in panels (**a**–**c**) show means + SEM. Different letters indicate statistically significant differences at $\alpha = 0.05$ (Reprinted from Tragust et al. (2013a) with permission of Cell Press)

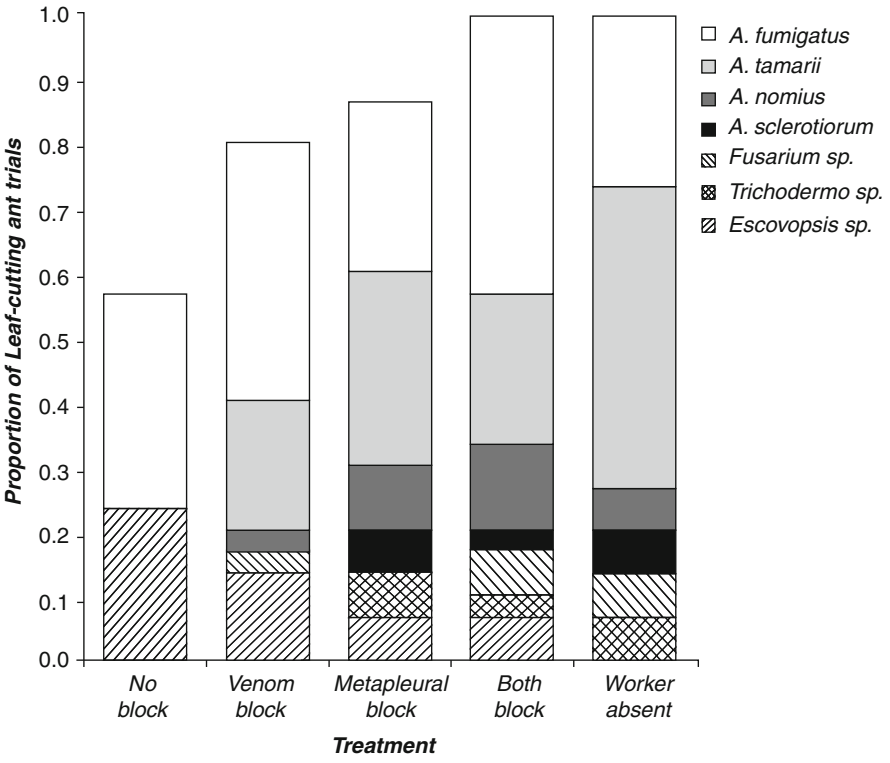

Fig. 8 Proportion of trials where foreign fungus overgrew leaf-cutting ant nest material, grouped by treatment. Foreign fungal species were *Aspergillus fumigatus* (*white*), *A. tamarii* (*light gray*), *A. nomius* (*dark gray*), *A. sclerotiorum* (*black*), *Fusarium sp.* (*leftward diagonals*), *Trichoderma sp.* (*cross-hatched*), and *Escovopsis sp.* (*rightward diagonals*) (Reprinted from Tranter et al. (2014) with permission of Springer)

Venom on the Cuticle and the Nest Surface as Externalized Immune Defense

Recently, venom components on the nest surface and on the cuticle of several species belonging to the genus *Apis* (*A. mellifera*, *A. dorsata*, *A. cerana*, and *A. andreniformis*) have been investigated with respect to their nesting ecology and environmental constraints (Baracchi et al. 2011). According to their nesting habits, the species can be divided into two groups: cavity-dwelling species (*Apis cerana* and *Apis mellifera*) and open-nesting species (dwarf honey bees *Apis andreniformis* and giant honey bees *Apis dorsata*). Using an analytical survey of medium-weight polar venom compounds, it was found that the major difference between these *Apis* species corresponds to nesting habit, i.e., between the cavity-dwelling and the open-nesting species. While the former have venom compounds on the cuticle, venom peptides are almost absent on those of *A. dorsata* and *A. andreniformis*.

Similarly, the antimicrobial venom compound melittin is present on the nest surface of both the cavity-dwelling species but not evident on the nest surface of the open-nesting giant honeybee and dwarf honeybee. This result is exactly what would be expected for the conditions favoring the evolution of external immune defense such as the use of externalized antimicrobial active venom suggested by Otti et al. (2014): i.e., a highly stable and confined environment with constant or high microbe pressure. In this context, it is interesting to note that extracts from the cuticle of social wasp species with paper nests show a higher antimicrobial activity than those of solitary species which excavate burrows, while extracts of solitary mud-nesting species show no antimicrobial activity at all (Hoggard et al. 2011) (Table 1). It might be argued that the environmental conditions found in excavated burrows and mud are much more variable than the conditions found in paper nests, thus not favoring the evolution of external immune defense. On the other hand, factors such as the relative contribution of social lifestyle and of phylogenetic relationships to the evolution of external immune defense clearly need to be considered and disentangled. For example, the primitive social hover wasps Stenogastrinae lack venom compounds on the nest surface, despite the fact that not a single species excavates burrows (Baracchi et al. 2012b). The following section of this chapter will explore whether life-history traits of social insects, namely, the high number of often closely related individuals living in high densities with frequent physical contacts, have indeed favored the use of antimicrobial active venom as external immune defense.

Table 1 Antimicrobial activity of cuticular extracts from several solitary, communal, and social wasp species

Species (family)	*n*	Sociality	Nest type	IC50 (±95 % CI)	n_r
Polistes humilis (Vespidae)	1077 (10)	Soc.	Paper	6.03 (±2.26)	28
Ropalidia plebeiana (Vespidae)	49 (2)	Soc.	Paper	7.58 (±5.91)	5
Bembix sp. (Crabronidae)	83	Com	Burrow	31.97 (±27.62)	6
Austroscolia sp. (Scoliidae)	47	Sol.	Burrow	158.27 (±152.82)	5 (3)
Cryptocheilus sp. (Pompilidae)	4	Sol.	Burrow	14.47	1
Pepsinae Sp1 (Pompilidae)	1	Sol.	Burrow	90.26	1
Abispa ephippium (Vespidae)	1	Sol.	Mud	No inhibition	1
Sceliphron laetum (Sphecidae)	5	Sol.	Mud	No inhibition	2
Delta sp. (Vespidae)	1	Sol.	Mud	No inhibition	1

n number of individuals (number of colonies for social species). Sociality: social (Soc.), communal aggregator (Com.), solitary (Sol.). IC50: mean equivalent surface area (mm^2) of wasp cuticle required to kill or inhibit 50 % of *S. aureus* growth. *nr* number of replicates per species (Reprinted from Hoggard et al. (2011) with permission of Plos Library of Science)

Social Lifestyle and the Evolution of Venom as External Immune Defense

Since the discovery of antimicrobial properties of hymenopteran venoms, it has been argued that the adaptive significance of this trait relies on protection from commensal pathogen infections during stinging events. However, experimental data supporting this hypothesis are lacking to date (Moreau 2013). Instead, researchers have started to shed light on the evolutionary significance of antiseptic venoms in social insects. Stow and coworkers (Stow et al. 2007) explored whether the evolution of sociality required the synchronous evolution of increased chemical defenses against pathogens in social bees. They found that the strength of antimicrobial compounds on the cuticle of bees was positively correlated to group size and genetic relatedness along a gradient of sociality ranging from solitary (*Amegilla bombiformis* and *Amegilla asserta*) and semi-social (*Exoneura robusta* and *Exoneura nigrescens*) to eusocial (*Exoneurella tridentata* and *Trigona carbonaria*). This indicates that the evolution of sociality was accompanied by the evolution of stronger antimicrobial compounds. The link between the levels of antimicrobial compounds on the cuticle and the levels of social complexity was also revealed by Hoggard and coworkers (Hoggard et al. 2011) in wasps. Besides trends of increasing antimicrobial activity along social complexity, within a single species, correlations between antimicrobial activity on the cuticle and both colony size and the level of within-colony genetic variation were also found (Hoggard et al. 2013). More precisely, in the paper wasp *Polistes humilis*, the effectiveness of antimicrobial activity on the cuticle increases with genetic diversity and decreases with colony size (i.e., the number of wasps forming the colony). It is most likely the venom that is responsible for the antimicrobial activity found on the cuticle, as venom components of bees and wasps are commonly found on the cuticle (see previous sections). Since the increase in antimicrobial strength on the cuticle found in the study of Stow and coworkers (Stow et al. 2007) was not linear, with the greatest increment being between smaller group sizes, it was suggested that selection pressure from microbial pathogens is so intense that even minimal sociality requires substantially stronger antimicrobials. Support for this hypothesis comes from the fact that even minimal societies such as those of the hover wasps *Metischnogaster drewseni*, whose colonies count a maximum of two to three females, have strong antimicrobial venoms (Baracchi et al. 2012b).

The same link between the strength of antimicrobial compounds and level of sociality has been established in both wasps (Hoggard et al. 2011) and bees (Stow et al. 2007), but information is lacking for ants. However, it is known that in fungus-growing ants, there is a positive correlation between the size of metapleural gland reservoirs, an important source of antimicrobial compounds on the cuticle of ants (Yek et al. 2013), and social complexity. The relationship between antimicrobials compounds and the level of sociality might thus hold throughout the social Hymenoptera.

Conclusion and Future Directions

This chapter has summarized the evidence that predatory and social lifestyles found in Hymenoptera have resulted in the increased use of venoms for defensive and offensive purposes. Intriguingly, a background antimicrobial function has been conserved or recruited in these venoms, indicating that microbial pressures have been important in shaping the evolution of the composition and the use of hymenopteran venoms. However, until recently, this has almost never been taken into consideration. Recent research has proposed that any heritable trait acting outside an organism and improving protection from pathogens or manipulating the composition of the external microbial community should be viewed as external immune defense (Otti et al. 2014). As outlined in this chapter, antimicrobial venom of Hymenoptera is frequently externalized for the purpose of self-sanitation, sanitation of related group members and the nest, and for the preservation of stored food. Thus, there is no doubt that antimicrobial venoms represent an important component of external immunity in Hymenoptera.

However, many facets of the ecological immunology of the venom remain insufficiently understood. External immune defenses come at a cost and are often tightly linked to the physiology of an organism and its internal immune system. Elucidating the costs related to the use of venom as external immune defense is thus required to clarify potential trade-offs in a more precise way. For example, it is known that the use of environment-derived antimicrobials as external immune defense in ants and bees reduces the expression of the internal immune response (Castella et al. 2008; Simone et al. 2009). Pros and cons of relying more on external rather than internal immunity clearly depend on different ecological and environmental factors, but this needs to be evaluated in more detail. Potential trade-offs between different external immune defense traits will also have to be taken into consideration, while recent advances in many technologies and analytical techniques will undoubtedly help researchers in this endeavor. However, insights from the fields of ecological immunology, chemical ecology, biochemistry, and molecular biology clearly need to be combined in order to complete our understanding of hymenopteran venom compounds and functions.

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Venom Use in Mammals: Evolutionary Aspects

12

Rodrigo Ligabue-Braun

Abstract

Mammals are recently accepted as venomous animals, with four orders having venomous representatives. These are Eulipotyphla (solenodons and some shrews), Monotremata (platypus), Chiroptera (vampire bats), and Primates (slow and pygmy slow lorises). Each of them has different strategies for using very diverse mixtures of toxic molecules. Venomous saliva is used by eulipotyphlans to paralyze and cache prey, and by chiropterans to avoid blood clotting in suitable prey, allowing continuous feeding. Monotremata use crural spurs to inject a highly painful secretion as a tool in sexual selection, while Primates lick an elbow gland, loading modified teeth with anaphylaxis-inducing venom. There is no homology between venomous systems in these different orders, making a common origin for all venom in Mammalia unlikely, even considering gaps in the fossil record. An emerging picture of complex interactions between cost of venom producing, specialized teeth for feeding and possible lack of benefits for venom in larger, stronger mammals may be able to justify the rarity of venom in this group. Both basic science and biotechnology are benefited as more knowledge accumulates about mammalian venoms.

Keywords

Mammals • Platypus • Bat • Loris • Shrew

R. Ligabue-Braun (✉)
Graduate Program in Cellular and Molecular Biology, Center for Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
e-mail: ligabue.braun@gmail.com; rodrigobraun@cbiot.ufrgs.br

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_20

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Introduction

Mammalian venoms may be considered a novelty, but they have not been discovered recently. For centuries, up to the 1940s, shrew bites were regarded as highly painful (and the animal itself was taken as evil and ill intentioned). Comparisons were drawn between shrews and venomous reptiles, including cobra and beaded lizards. However, with advances in microbiology and the advent of antibiotics, effects originally attributed to shrew saliva were attributed to the action of microorganisms from the animal's mouth. It took 50 years for this topic to be rediscovered, or, more accurately, rediscussed. Unfortunately, Dufton's seminal work (Dufton 1992) remained largely ignored by mainstream zoologists (and biologists as a whole). The emergence of more solid evidence on many mammalian venoms, two decades later prompted new investigation of the subject (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

There are four mammalian orders with known venomous representatives, as recognized today. These comprise solenodons and some species of shrews (Order Eulipotyphla), platypuses (Order Monotremata), vampire bats (Order Chiroptera), and slow lorises (Order Primates). The amount of knowledge regarding each class varies greatly. There is also great variation in the strategies in which the venoms are employed. These secretions are used to immobilize prey, to facilitate feeding, for predator defense, and for sexual selection. In this chapter, historical aspects and specifics of the venoms' toxicity will be presented first. Subsequently, evolutionary mechanisms that led to each of these venom-use strategies will be discussed.

Venomous Mammals: Overview

Eulipotyphla

The eulipotyphlans include the majority of venomous mammals. These are the American short-tailed shrew (*Blarina brevicauda*), the Hispaniolan solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*), and the Mediterranean water

shrew (*Neomys anomalus*). Some evidences support venom in the Cuban solenodon (*Solenodon cubanus*) and the Canarian shrew (*Crocidura canariensis*), while circumstantial evidence may point to venom in the European mole (*Talpa europaea*). Despite being omnivores, the eulipotyphlans were formerly included in the "wastebasket" taxon Insectivora (Greek for "eaters of insects"). This was a major misnomer, since these animals prey on varied invertebrates and on vertebrates of the same, or even larger, size as themselves. The venom asset of these mammals is found in their saliva, produced in enlarged granular submaxillary salivary glands. Venomous saliva is also found in vampire bats, albeit of different composition (see below). The most extensively studied eulipotyphlan saliva is the one from *B. brevicauda*, since many factors hinder studies with other species, ranging from difficulties in keeping these animals in captivity (shrews) to their endangered status (solenodons).

The European shrew probably was the first mammal to be historically recorded as venomous. In 1607, Reverend Topsell's "History of Four-footed Beasts" described the animals as cunning and cruel, pretending to be gentle and tame, but desiring to hurt anything with a deep, deadly, bite. In Europe, shrews have been associated with malignancy, depravity, wickedness, and taken as signs of ill omen. The Latin name for the European shrew, *Sorex araneus*, and the French common name, *musaraigne*, derive from the Latin word for spider (*aranea*). In North America, shrews were considered mostly harmless, and their dangerous status was relegated to folklore, while the natives of the Caribbean Islands regarded solenodons as venomous. Solenodons and shrews had their venomous saliva scientifically examined almost simultaneously. In 1877, Gundlach studied bites of Cuban solenodons (*S. cubanus*), comparing them to bites from venomous snakes, while Maynard, in 1889, made a case report on the effects of an American short-tailed shrew (*B. brevicauda*) bite. From 1942 to the 1960s, the saliva toxicity of various eulipotyphlans was tested on animal models. However, this line of research seemed abandoned until 1992, when a major review with new data (Dufton 1992) brought the subject back into the spotlight. In the 2000s, the identification of the *B. brevicauda* toxin (Kita et al. 2004) and the uncovering of fossil shrews with envenomation apparatus (Cuenca-Bescós and Rofes 2007) once again emphasized this aspect of mammalian physiology.

Bites from shrews are considered uncomfortable to humans, with personal perceptions ranging from no detectable effect to an immediate burning sensation, swelling, and impossibility of using the affected member for days (Ligabue-Braun et al. 2012). One of the first observations made about the Cuban solenodon bite was that the lower incisors caused inflammation at wound entry, while the upper incisors had no effect, something considered common by natives. This was the first observation that the submaxillary glands were the production site of venom (Dufton 1992). Solenodons have gutter-like grooves in their inferior incisor teeth that allow saliva flow to the bite-induced wound. Shrews lack this modification. When present (as in the Eurasian shrew), there is only a slight concavity in the incisors (Fig. 1).

Testing *Blarina*, *Neomys*, and *Solenodon* submaxillary extracts on mice, rabbits, and cats established that the venomous saliva causes general depression, breathing disturbance, paralysis, and convulsions. Even though small vertebrate prey (especially mice and voles) are an important part of some eulipotyphlan diets,

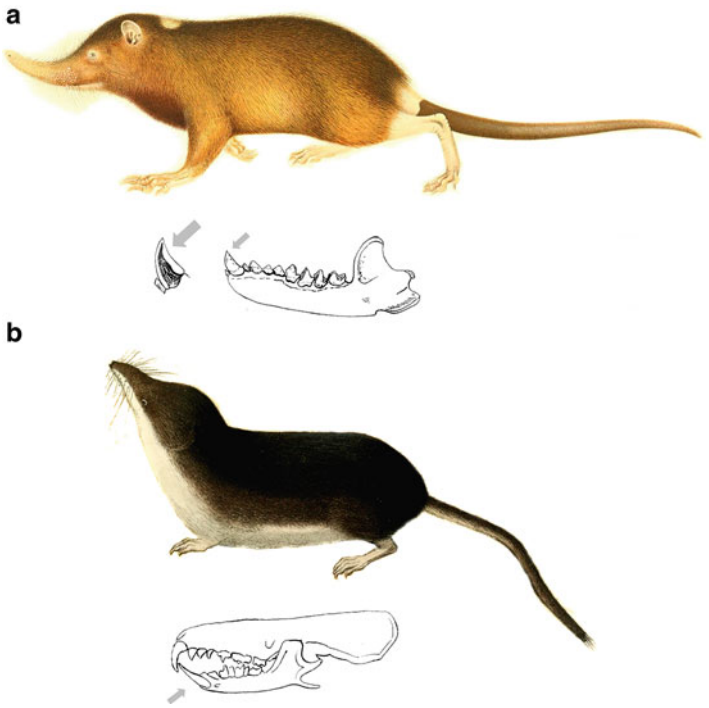

Fig. 1 Venomous Eulipotyphla. (**a**) General aspect of a Solenodon (*Solenodon paradoxus*). The detail highlights the grooved incisor teeth. (**b**) General aspect of a Eurasian shrew (*Sorex araneus*). The detail highlights the slightly concave incisor teeth (Author's own artwork, incorporating images in the public domain from "Solenodon paradoxus" by GM Allen, "Règne animal" by G Cuvier, and "Faune des vertébrés de la Suisse" by V Fatio)

invertebrates account for most of the animals' nutrition. Based on that, tests of *B. brevicauda* with experimental insect prey (such as roaches and crickets) revealed that its saliva has immobilizing effects, with these immobilized insects being stored for later consumption. In natural conditions, *B. brevicauda* caches a varied array of preys in a comatose state. These include, besides insects, snails, earthworms, and small mammals (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

The active compound in the saliva was thought to be a neurotoxin, based mainly in some similarities between shrew and cobra venoms proposed in the late 1940s (Ligabue-Braun et al. 2012). However, no resemblance was found (Lawrence 1945; Dufton 1992). This neurotoxin-targeting search, coupled with difficulties to work with pure *B. brevicauda* submaxillary gland extracts, hindered further research into this topic until the 2000s. In 2004, it was found that the major toxic component in *B. brevicauda* saliva is blarina toxin (BLTX) (Kita et al. 2004). Still, other, unidentified synergistic components may be acting in the venom. BLTX is an N-glycosylated kallikrein-like protease of 253 amino acids, with heterogenous glycoforms. This toxin releases bradykinin from kininogens and would be

responsible for the effects observed in experimental animals (dyspnea, hypotension, hypokinesia), since bradykinin is an inflammation mediator that acts in increasing vascular permeability and lowering blood pressure.

Monotremata

There is only one venomous Monotremata species, the platypus (*Ornithorhynchus anatinus*). This egg-laying mammal has semifossorial, semiaquatic habits, living in rivers and streams in the eastern coast of Australia. Both males and females are born with spurs in their hind legs, but only the former maintain them for life. These keratinized spurs are connected to the crural glands, which produce venom, forming the venom-injecting structure known as the crural system (Grant and Temple-Smith 1998). The crural glands are found in the dorsocaudal sides of the abdomen, and derive from sweat glands (Whittington and Belov 2016; Fig. 2).

Platypus envenomation was first recorded in the scientific literature in 1818, and detailed anatomical description, including venom use and tests on domestic animals, followed. From 1935 to the 1960s, there seems to be no records on this subject. In 1968, however, a major monograph on platypus detailed the toxic properties of the crural secretions. Some envenomation case reports have been published since but have been normally treated more as a curiosity than as a real medical issue (Ligabue-Braun et al. 2012). From 1995 onwards, more biochemical characterizations of the venom became available, and the Platypus Genome Project (Warren et al. 2008) allowed a much more detailed inspection of this secretion.

Platypuses have been hunted for their fur by Australian colonists, who sometimes were victims of envenomation. In humans, all cases involved hands or wrists. The venom, injected by repetitive jabbing of spurs from both hind legs pressed against one another, causes immediate acute pain and swelling and requires anesthetic blockade combined with intravenous narcotic infusion as regular analgesic treatment is ineffective. The envenomation symptoms may persist for a long period, from

Fig. 2 Venomous Monotremata. General aspect of a platypus (*Ornithorhynchus anatinus*). The detail highlights the crural spur (Author's own artwork, incorporating images in the public domain from "Genera mammalium," by A Cabrera)

2 weeks to several months. In test animals, swelling and tenderness at the site of injection were observed, followed by decrease in blood pressure, respiratory distress, and death (Whittington and Belov 2007).

During the mating season, male platypuses are frequently found with punctures in their bodies, despite attacks among platypus being rarely observed. The crural glands show cyclic activity, becoming highly active during the mating season, producing venom to be delivered by the channeled spur (Grant and Temple-Smith 1998). Due to this cyclic activity and the protected status of platypuses, studies on their venom composition have been hindered until recently. The venom is a complex mixture of C-type natriuretic peptides, defensin-like peptides, nerve growth factors, isomerases, hyaluronidases, proteases, and other, uncharacterized proteins (Whittington and Belov 2007).

Four major components of the venoms have had more in-depth characterization (Whittington and Belov 2007; Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015) but their exact functions have not been established (Whittington and Belov 2016). C-type natriuretic peptides differ from A and B natriuretic peptides, which act in controlling blood pressure, by lacking natriuretic activity, suggesting other actions for these peptides. They are the most biologically active peptides in the platypus venom, and may be responsible for envenomation signs (such as hypotension). These peptides seem able to disrupt membranes and interact with putative nociceptors. Defensin-like peptides are structurally similar to β-defensins but lack sequence and functional similarity with them. These are the most abundant peptides in the venom and could cause pain, possibly by synergistic action with venom nerve growth factors. These factors are also devoid of their classical function, having a putative immunogenic effect. C-type natriuretic peptides and defensin-like peptides from platypus venom also show isoforms with either L- or D-amino acids in specific positions. This is due to an L-to-D-amino acid-residue isomerase. Though not confirmed, the function of these D-residues seem to be resistance to proteases while in the crural gland.

Chiroptera

Among Chiropterans, only a subfamily of New World leaf-nosed bats (Phyllostomidae) holds venomous representatives. These are the vampire bats from the Desmodontinae subfamily. They are found from Mexico to southern Argentina and comprise the common vampire bat (*Desmodus rotundus*), the rarer hairy-legged vampire bat (*Diphylla ecaudata*), and white-winged vampire bat (*Diaemus youngi*). The saliva of these bats has anticoagulant properties and is part of many other adaptations that allow these animals to feed on blood only, including razor-sharp teeth (Schondube et al. 2001; Tellgren-Roth et al. 2009; Fig. 3).

Venomous bats satisfy the criteria for producing venom, i.e., a secretion produced in a specialized gland in one animal and delivered to a target animal through the infliction of a wound, containing molecules that disrupt normal physiological processes so as to facilitate feeding or defense by the producing animal (Brodie 2009;

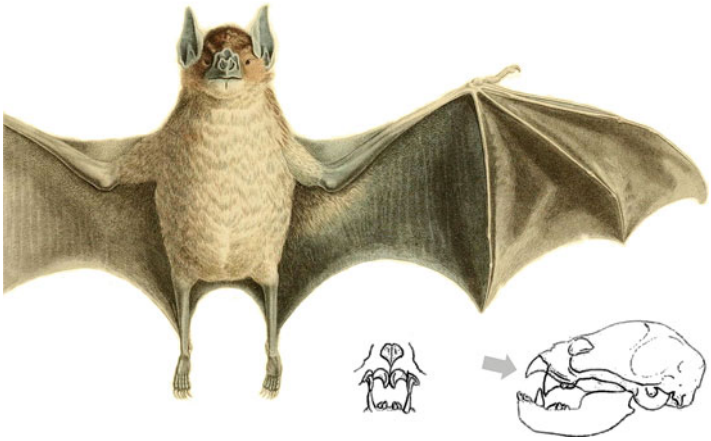

Fig. 3 Venomous Chiroptera. General aspect of a common vampire bat (*Desmodus rotundus*). The detail highlights the modified sharp teeth in front and side view (Author's own artwork, incorporating images in the public domain from "Voyage dans l'Amérique Méridionale," by AD d'Orbigny)

Fry et al. 2009). However, the large majority of vampire preys do not perish from the venom, which causes only a minor discomfort. In this regard, vampire bats resemble parasites in their feeding behavior (Delpietro and Russo 2009), since the physiological disruption facilitates feeding while keeping the prey alive, ensuring continuous nutritional supply for the bats (Fry et al. 2009).

For centuries prior to the discovery of vampire bats, Europe had legends of supernatural blood-sucking entities (Ligabue-Braun et al. 2012). Serendipitous crossing of such folklore with the growing reports on hematophagous bats from South and Central America from 1498 onwards led to the association of these animals with the myth of the vampire, summited by the publishing of "Dracula" (despite the fact that bats are mostly unmentioned in Bram Stoker's book).

Vampire bats have characteristic feeding bites, which are sharply circumscribed, crater-like, 4 mm wounds inflicted onto the attacked animal's bare skin. The anticoagulant saliva allows bats to ingest a continuous flow of blood for up to half an hour, through a piston-like motion of the tongue. In vivo comparisons have shown that a bat-inflicted wound may bleed from 180 to 480 min, while an equivalent, blade-induced wound bleeds for about 15 min (Tellgren-Roth et al. 2009).

The three species of hematophagous bats prey upon different animals (Tellgren-Roth et al. 2009). The common vampire bats (*Desmodus rotundus*) mostly feed on farm animals, such as cattle, horses, goats, pigs, and sheep. Less commonly, they feed on humans, poultry, and wild prey. The white-winged vampire bats (*Diaemus youngi*) feed mostly on birds but are also able to feed on mammals, while the hairy-legged vampire bats (*Diphylla ecaudata*) feed exclusively on birds.

The free bleeding of bat-inflicted wounds led many researchers to propose that some kind of anticoagulant should be present in the saliva. In 1966, one such compound, a plasminogen activator, was identified, followed in the 1990s by molecular characterization of four activators and a factor Xa inhibitor. In the 2010s, yet another plasminogen activator was identified (Ma et al. 2013), along with the molecular characterization of the fXa inhibitor (Francischetti et al. 2013; Low et al. 2013).

Bat venomous saliva has tissue-type plasminogen activators and a lactoferrin, while other components may still be discovered. The plasminogen activators (originally identified in 1966 but still being unfolded into different types) convert the plasmin proenzyme to its active form, which is able to degrade blood clots. While the rarer vampire bats have only one type of plasminogen activator in their genomes, the common vampire bat has five (Tellgren-Roth et al. 2009; Francischetti et al. 2013; Low et al. 2013).

The tissue-type plasminogen activator molecule has five domains: finger, epidermal growth factor, kringle 1, kringle 2, and serine protease. Only the *D. ecaudata* plasminogen activator has all five domains, with the other two species having smaller chains with domain deletions (Tellgren-Roth et al. 2009; Ma et al. 2013). These chain variations, combined with variable glycosylation structures (one *O*- and two *N*-glycosylation sites) alter binding properties of vampire bat plasminogen activators compared to tissue-type plasminogen activators.

The second type of anticoagulant from vampire bat saliva is draculin (Francischetti et al. 2013; Low et al. 2013). This modified lactoferrin is a noncompetitive, tight-binding inhibitor of activated factor X from the coagulation cascade. Factor Xa is the only enzyme that converts prothrombin into thrombin (a key point in the blood coagulation process). Draculin action is dependent on correct *N*- and *O*-glycosylation, and a mixture of draculin glycoforms are proposed to modulate the degree of fXa inhibition. As with many other vampire bat studies, draculin has been inspected only in *D. rotundus* so far.

Since the feeding bites from hematophagous bats are considered painless, it has been proposed that their saliva may also have an anesthetic. However, vampire bats are known to learn how to properly bite prey by trial and error. So far, there is no concrete evidence for this putative anesthetic.

Primates

The nocturnal prosimians slow loris (*Nycticebus coucang*, *N. bengalensis*), Kayan slow loris (*Nycticebus kayan),* and pigmy slow loris (*N. pygmaeus*) are the venomous representatives of the Primates order (Rode-Margono and Nekaris 2015). They inhabit trees in Southeast Asia and Western Indonesia. They are unique in their mode of toxin delivery, since unrelated body parts produce and inject the venom. This venom is synthesized in the brachial gland, located in the ventral, almost hairless, side of the elbow. Then, by licking of the gland, the secretion is mixed with saliva and loaded into the toothcomb, a specialized compression of the needle-like canines

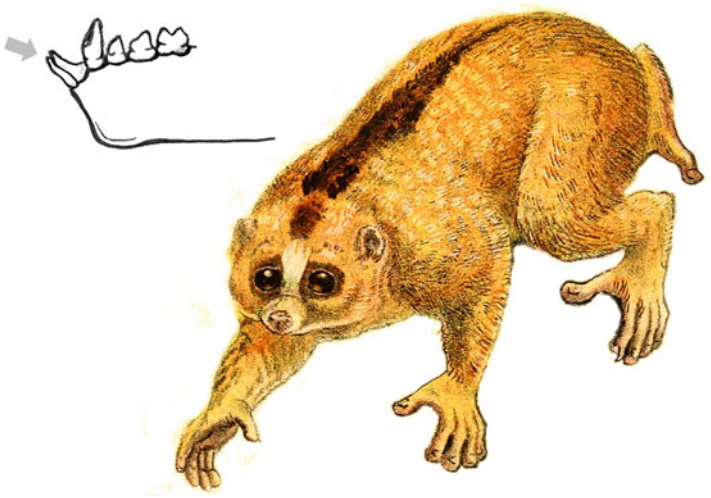

Fig. 4 Venomous Primates. General aspect of a slow loris (*Nycticebus coucang*). The detail highlights the modified tooth comb (Author's own artwork, incorporating images under Creative Commons license by Kathleen Reinhardt, and in the public domain from "A handbook to the primates" by HO Forbes)

and incisors of the loris jaw (Hagey et al. 2007; Fig. 4). Exhibition of the elbow, by positioning of their front hands above the head, and intense spreading of the venom on the head are also taken as indications of venomousness. Most (if not all) generally accepted definitions of a venomous animal state that the venom producing site and the delivery (or injecting) organ must be directly connected. This is not the case with the primates, which are only now becoming accepted as venomous (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

Folklore in Thailand holds lorises as venomous animals capable of causing intense pain and death. However, human envenomation is rarely reported. There are only three records in the medical literature (a man bitten by his pet *N. coucang*, a pregnant zookeeper bitten by a *N. pygmaeus*, and a researcher bitten by a *N. kayan*), and anecdotal evidence from manuals for zookeepers and wildlife caretakers (Madani and Nekaris 2014). The animal bite causes some effect besides the laceration itself – symptoms that do not differ from anaphylaxis. These include pulsating pain, hypotension, extremity cyanosis, and hematuria. Researchers working in close contact with lorises develop allergies to the venom. Despite its ability to cause anaphylactic shock, this seems to be only an incidental effect of the venom (Hagey et al. 2007).

There is ongoing research aiming to define the main physiological role of the loris brachial gland secretion, which may act synergistically with the animal's saliva (Rode-Margono and Nekaris 2015). Venom use for prey capture is not supported, while use in intraspecific competition seems plausible. Predator and ectoparasite defense are the most well-supported ecological roles for the venom.

Despite being a highly complex mixture, containing dozens to hundreds of compounds, the main toxic component of the venom has been identified as the

brachial gland exudate protein (BGE protein) (Krane et al. 2003). It is a heterodimeric protein (17.6 kDa), with the α-chain (7.8 kDa) and β-chain (9.8 kDa) linked by two disulfide bridges. All studied lorises have two BGE isoforms, due to variable β-chains. The BGE protein is highly similar to the major cat allergen, Fel d 1. Both share the uteroglobin protein fold, are disulfide bound, and have alternate β-chains. The uteroglobin fold is related to transport of hydrophobic molecules (such as steroid hormones) and calcium binding. Regardless of being poorly understood, uteroglobins are postulated to act as boxes, being able to open and close according to physiological conditions, loading, carrying, and delivering hydrophobic cargo (Hagey et al. 2007). The similarity to a feline allergen reinforces the possible cross-reactivity of loris venom instead of a de facto venomous role (at least for humans) (Ligabue-Braun et al. 2015).

Mammalian Venom Evolution: Shadows of the Past or Rare Recurrences?

As can be observed from the mammalian phylogenetic tree (Fig. 5), orders with venomous representatives do not cluster together, having no obvious common origin. This observation prompts the question: Why are venomous mammals so rare? Moreover, what is the utility of the extant venom, being so scarce among this animal class? Since each order's venom is different, the following segment will describe peculiarities and strategies in which these mammalian groups employ venoms, discussing current evolutionary hypotheses regarding their origins.

Venomous eulipotyphlans have been compared to venomous reptiles long before the identification of BLTX. Solenodons and shrews were studied taking snakes as reference (Dufton 1992). In 1942, when identifying that shrews had modified salivary glands responsible for the venom production, like snakes, Pearson also noted that in the latter the parotid glands are modified to produce venom, while in the former, the submaxillary glands are the venom source. Despite this important difference between the two cases, there are indeed venomous reptiles with venom-producing submaxillary glands (including the Gila monster *Heloderma suspectum* and the Mexican beaded lizard *H. horridum*). With the purification of BLTX, it was confirmed that these cases were indeed linked (Kita et al. 2004). The Gila toxin (GTX) and BLTX have similar effects on prey and are similar (34% identical). Horridum toxin also has high sequence similarity with BLTX (32%). The reptilian and mammalian kallikreins underwent convergent transitions to venomous ones, with similar, nonhomologous residue insertions that increased the protein flexibility, altering loop lengths, polarity, and surface charges. Both GTX and BLTX started from independent serine proteases that had locally different alterations generating globally similar, toxic, structures (Aminetzach et al. 2009; Fig. 6). Blarinasin, another kallikrein-like protein from shrew saliva is not toxic on tested animals, suggesting that small differences, including glycosylation heterogeneity, may play key roles in their toxicity.

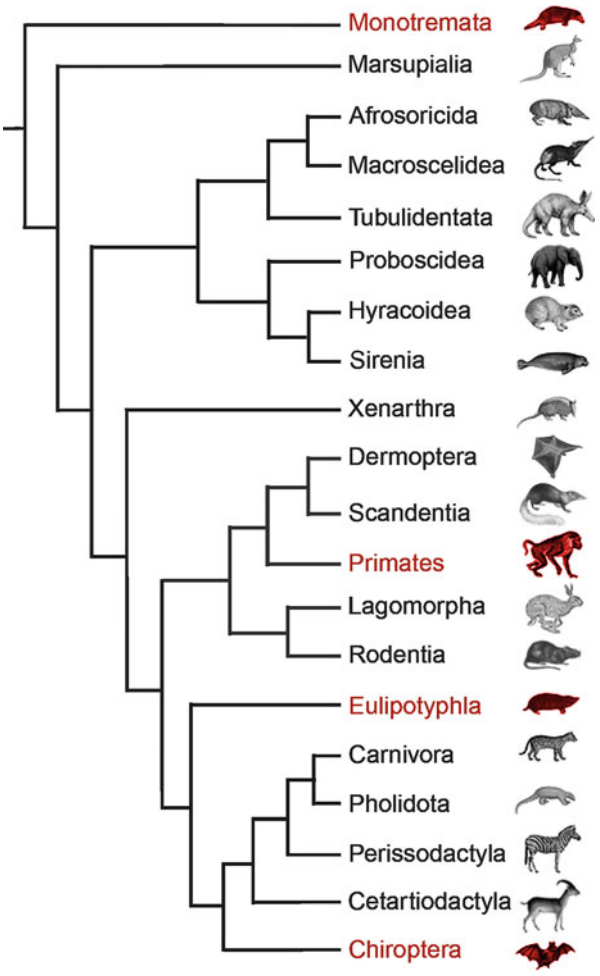

Fig. 5 Mammalian phylogeny highlighting orders with venomous representatives (in *red*). Please note that there are alternate versions for the evolutionary history of Mammalia (Author's own artwork, based on Springer et al. 2004)

Regarding the venom function in Eulipotyphla, Furió et al. (2010) defined an ongoing debate as "hunting big or hoarding small." As part of an adaptive winter profile, shrews cache various preys in a comatose state. Other adaptions include elaborate nests, stable thermal regime for foraging, and reduced activity during periods of cold. Within this framework, venom would be an asset to sustain a living hoard when hunting is difficult, especially in cold winters. The high metabolic rate of shrews would make this ability very relevant. The use of eulipotyphlan venom as a paralyzing, conservative agent is thus taken as support for the "hoarding small" hypotheses. The "hunting big" hypothesis proposes that venom is a tool to overcome bigger prey. According to Dufton (1992), vertebrate food is of major importance for eulipotyphlans, and this kind of prey is larger and more dangerous to subdue than their power-to-weight ratio would allow, thus making venom necessary. There is no specialized venom delivery apparatus in extant shrews, which have only a concavity

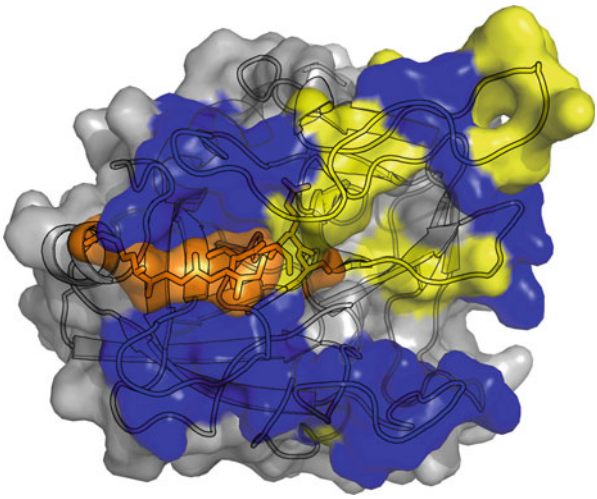

Fig. 6 Structural model of blarinatoxin, BLTX, with regulatory loops colored in *blue* and insertions that convergently evolved towards toxicity in *yellow*. A substrate is shown in *orange*. Model built based on PDB ID 2ZCK, Ménez et al. (2008) (Author's own artwork)

on the surface of their first incisors. However, the discovery of an extinct giant shrew (*Beremendia* sp.) with an envenomation apparatus (grooved incisors similar to those from solenodons) (Cuenca-Bescós and Rofes 2007) seems to favor Dufton's proposal. These grooves would act as a channel, directing saliva from the submaxillary glands to wounds inflicted on the prey, as seen in solenodons. However, alternative explanations, based on paleoenvironmental reconstructions, propose that *Beremendia* lived in a highly unpredictable environment and that their prey consisted mostly on gastropods and coleopterans (Furió et al. 2010). Most likely, venom is used by Eulipotyphla in both ways, including combining them to hoard larger prey. Secondarily, venom use in intraspecific competition has been observed among captive solenodons.

In his model for venomousness in mammals, Dufton (1992) proposed that the earliest eutherian mammals had morphologies that resemble extant hedgehogs and shrews. These early eutherians developed during the late Cretaceous (66–144 millions of years ago), and would form a basal group for extant mammals (Rode-Margono and Nekaris 2015). Eulipotyphlans per se are not ancestral in mammalian phylogeny, but in this proposal, they would be the extant mammals that retained the most from these ancient eutherians. The current distribution of venomous eulipotyphlans, covering Asia, Europe, North and Central America, would support this view of a more widespread occurrence of venomous mammals in their evolutionary past. Since these ancestral mammals were small and not fully homeothermic, foraging efficiency would act as a selective pressure, while the use of venom would bestow a selective advantage on them (Rode-Margono and Nekaris 2015). Dufton also observed that extant venomous eulipotyphlans almost exclusively co-occur with flightless birds. This scenario would somewhat resemble their origins sharing habitats with dinosaurs, suggesting that beyond egg-eating, larger flightless birds (or dinosaurs) could be targets for venom (either predatorily or defensively). Once

their diet shifted to invertebrates, venom would become less useful, being retained in only a few species. This is an especially cumbersome hypothesis, since extant venomous Eulipotyphlan are very successful at using venom to prey on invertebrates (Folinsbee 2013).

Despite the well-supported occurrence of venom in the extinct giant shrew *Beremendia* (and possibly in the solenodon relative *Nesophontes*), other evidences from the fossil record are still being disputed. The discovery of a Palaeocene eutherian mammal with canine grooves, phylogenetically distant from Eulipotyphla, prompted its classification as a venomous mammal (Fox and Scott 2005). This animal, *Bisonalveus browni*, was proposed to use its grooved teeth to deliver toxic saliva, resembling solenodons. However, the occurrence of grooved teeth alone has been deemed insufficient and inadequate to support the occurrence of true venom delivery apparatus in extinct mammals. Orr et al. (2007) and Folinsbee et al. (2007) cited numerous examples of extant mammals with grooved teeth and no sign of venom. These include suiforms, coatis, lemurs, and primates. The grooving in their teeth seems to act as a structural reinforcement of the dentary structure, unrelated to venom delivery. Both works conclude that the traditional comparative method alone could not ascertain if a primitive mammal was venomous without slipping into "false positives," i.e., if the structure (grooved teeth) is related to function (venom delivery), all extant mammals with this anatomy would be expected to be venomous, which is not the case. Additionally, except for solenodons, all other extant venomous mammals lack truly grooved teeth. The mammalian masticatory apparatus, however, is considered highly sophisticated, enabling a wide range of feeding strategies. This, alone, could render venom use redundant (Folinsbee et al. 2007). As observed by Rode-Margono and Nekaris (2015), this seems to be the case, since the venomous eulipotyphlans are exceptions to the general pattern in mammalian diets. While many orders are chiefly herbivorous or insectivorous with small prey relative to predator body mass, the carnivorous orders are large and able to overcome their prey by sheer strength.

In a recent phylogenetic construction of Eulipotyphla phylogeny, Folinsbee (2013) observed that *Neomys*, *Solenodon*, and *Blarina brevicauda* are phylogenetically distant, with the *Solenodon* lineage diverging around 80.5 mya and the ancestors of *Neomys* and *Blarina* diverging around 16.5 mya. The author observed that, if venomousness was an ancestral condition of Eulipotyphla, at least nine convergent losses of venom would be required to explain the obtained tree. On the other hand, if venom evolved convergently in different eulipotyphlans, only three unique acquisitions would be required. Still, venom is rare in this group, occurring in no more than 2% of extant species.

Toxic saliva is not exclusive to shrews and solenodons. Vampire bats use their venomous secretion to be able to feed on the continuous blood flow from a sharply cut wound. Vampire bat saliva, however, is just part of a highly specialized physiology, as a reflection of blood being their sole source of hydration and nutrition. Other adaptions include a modified gastrointestinal tract, which allows the ingested blood to enter the intestines prior to entering their tubular stomachs (due to a T-shaped gastroesophageal-duodenal junction). When reaching around half their

weight in ingested blood, most of the water is eliminated in a process known as instant diuresis, which leads to the highly nitrogenous blood remains being processed with almost no water. Their high capacity for concentrating urea in the urine makes vampire bats physiologically equivalent to desert mammals. Hematophagous chiropterans feed almost exclusively on the proteinaceous moiety of the ingested fluid, with the carbohydrates being almost unused, and sucrase and maltase being absent in their gastrointestinal tracts (Schondube et al. 2001). Another physiological characteristic of these animals is that they have no adipose tissue for storage, making them dependent on daily blood meals and reliant on a highly ordered social system, in which fed animals are able to regurgitate blood into the mouth of individuals that are unsuccessful or unable to prey for themselves. Their sharp teeth and anticoagulant saliva work as to facilitate the tongue-directed flow of blood to the animals' mouth. The tongue does not act licking up the blood, but rather acting like a piston. For this method to work, blood cannot coagulate, as to guarantee free flowing from the prey to the predator.

Vampire bats form a subfamily, Desmodontinae, in the Phillostomidae family. This family is considered to have the most diverse feeding habits among all mammal families. They include nectarivory, omnivory, frugivory, carnivory, and hematophagy (the latter deriving from insectivory) (Schondube et al. 2001). The three hematophagous species have different preferred preys, and this reflects the evolution of their salivary anticoagulants. The fibrin specificity and susceptibility to plasminogen activator inhibitor 1 of chiropteran plasminogen activators has been altered by gene duplication, domain losses, and further sequence evolution (Tellgren-Roth et al. 2009; Fig. 7). *D. ecaudata* has a single copy of plasminogen activator, which is similar to the one found in other mammals and feeds only on birds. *D. youngi* plasminogen activator lacks the kringle 2 domain, having increased

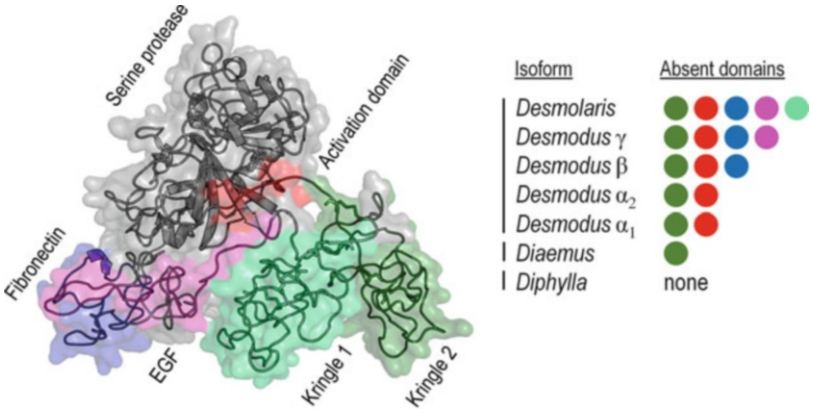

Fig. 7 Structural scheme of plasminogen activators from vampire bats. Absent domains in each protein are color-coded, while isoforms from each species are clustered together. Depicted based on Tellgren-Roth et al. (2009), model built based on PDB ID 4DUR, Law et al. (2012) (Author's own artwork)

fibrin specificity. This bat is more generalist, feeding on birds and mammals. The plasminogen activators in *D. rotundus* went through rounds of gene duplication and domain loss, creating versions with decreased sensitivity to plasminogen activator inhibitor 1 and enhanced ability to feed only on mammalian blood (Tellgren-Roth et al. 2009). Differences in glycosylation also take part in the improved anticoagulatory activity, since the clearance of some chiropteran plasminogen activators are up to four times slower than those observed for tissue plasminogen activators.

Only chickens have been observed to die of hemorrhage after vampire bat attacks. Other prey do not succumb to their bites or saliva, prompting arguments against the inclusion of bats as venomous animals (Ligabue-Braun et al. 2012). Indeed, their saliva facilitates feeding by disrupting regular prey physiology while ensuring its survival for the continuous supply of nutrition for the bat. The highly specialized saliva of Chiroptera is not homologous to Eulipotyphla venom, with the involved teeth being different as well as the molecules involved in the toxicity. Additionally, the emergence of hematophagy in bats is considerably more recent in evolutionary terms than the speciation of solenodons and shrews with envenomation apparatus. These observations highlight the emergence of venom more than once in the history of mammals.

Male platypuses (Monotremata) employ the secretion of their crural system in a different way to the venomous saliva from Eulipotyphla or Chiroptera, which is used in prey acquisition and feeding facilitation, respectively. The use of the glands and spurs has been proposed to take part in multiple behaviors in these animals, from helping climb riverbanks to waterproofing the fur (Grant and Temple-Smith 1998). However, their true usage is to act as a weapon in male-male competition for females, taking part in sexual selection. Adult male platypuses largely avoid each other and have testicular and crural gland size increases in the mating season, in which males become aggressive. In this season, it is common to find males with spur punctures, especially in their tails. Grant and Temple-Smith (1998) used this evidence to propose a polygynous mating system for platypuses. In this system, male interactions direct the access to females, something that would justify the retention of the crural system in former. Only one other Monotremata representative, the short-beaked echidna (*Tachyglossus aculeatus*), had its crural apparatus examined (Krause 2009). Both genders of these animals have degenerate crural spurs, with the males having cyclic growth of the crural gland in accordance to mating season. However, they are unable to use their spurs aggressively or to support the structure on their tibia during attacks. The seasonal growth cycle would suggest a role as scent gland, but its real function is still uncertain.

Monotremata are the sole remaining mammals from the class Prototheria, the first to diverge from other mammals (around 166 Mya). Platypus, in special, have anatomical features that are closely related to reptiles (similar ribs and pectoral girdle), despite being furred homeotherms. These mixed characteristics are reflected in their genome, with a large amount of reptilian-like genes, and taken as a possible link between reptiles and therians (Whittington and Belov 2016). Likewise, platypus venom has many similarities with reptilian ones, via convergent evolution

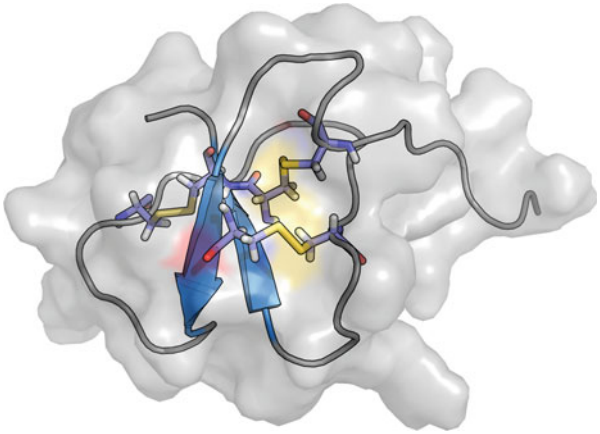

Fig. 8 Structure of defensin-like peptide 2, DLP-2, from platypus venom (PDB ID 1ZUE, Torres et al. 2005). The three characteristic defensin disulfide pairs are highlighted (Author's own artwork)

(Whittington et al. 2008). In both cases, defensin-like peptides, C-type natriuretic peptides, and nerve growth factor gene families were duplicated and then co-opted for toxic purposes (Warren et al. 2008). Many of the proposed "venom genes" are also expressed in non-venom tissues (i.e., outside the crural gland), while the natriuretic peptides and nerve growth factors from venom are also expressed in females, suggesting additional roles for these peptides. As observed in reptiles, there is an ongoing debate on whether putative toxins expressed in multiple tissues constitute true toxins (please see ▶ Chap. 4, "A Critique of the Toxicoferan Hypothesis" for more details). A transcriptome of platypus venom gland labeled 88 toxin genes when filtered against transcriptomes of nonvenomous tissues (Whittington et al. 2010). So far, only defensin-like A (Fig. 8) is considered a crural-gland exclusive peptide (Whittington and Belov 2009).

As with eulipotyphlans, fossil evidence has been interpreted as signs that crural spurs were widespread among primitive mammals. The proposed role for the crural system would be in defense against larger predators, dinosaurs in particular (Hurum et al. 2006; Kielan-Jaworowska and Hurum 2006). Once again, this proposition is based on comparisons with extant mammals, of which only one species has a venom-delivery apparatus involving crural glands and spurs. Still, true monotreme fossils are rare and consist of tooth and jaw fragments, not allowing certainty in defining ancestral monotremes as venomous. Considering the conserved (vestigial or functional) crural systems in extant platypuses and echidnas and some shared elements between their glandular secretions, it is possible to propose that their ancestral species were also venomous (Whittington and Belov 2016). The fact that spurs (functional or vestigial) are present in both sexes in extant Monotremata raises the possibility that their original purpose was in defense, especially in confronting large predators, from dinosaurs to large mammals (from the Jurassic to Pleistocene). It has been proposed that, once this selective pressure was no longer present, the crural system was co-opted to a reproductive context. This change would justify the maintenance of energetically expensive venom production as a sexually dimorphic trait (Whittington and Belov 2016).

There are many cross-taxa examples of convergent venom evolution (Fry et al. 2009), and the very unusual mammalian ones are no exception. Most toxins in mammalian venoms (with the possible exception of BGE protein) are products of this type of process, being similar to toxins found in other animal groups. Rapid effect, be it fast prey immobilization or quickly occurring pain, is a major requirement for a venom. This is proposed to be one of the main factors that limit what kinds of protein may be co-opted for toxicity. There is only a small group of proteins that recurrently develop into toxins once their genes are duplicated. However, this is not the sole process acting on mammalian venoms, since mutations in regulatory or coding regions and alternate splicing has been shown for platypus venoms, and alternate glycosylation has been related to variable activity in shrew and vampire bat venoms.

The order Primates displays a unique venom delivery system, unlike any other present in venomous animals. Currently four species in the *Nyctycebus* genus are recognized as being venomous. They load needle-like modified teeth with the secretion of an elbow gland, possibly mixing it with saliva, thus establishing a venom delivery apparatus from unrelated body parts. Many hypotheses have been tested to ascertain the ecological role of this venom, as reviewed by Rode-Margono and Nekaris (2015). Aiding in feeding seems unlikely, since their diet consists of fruits, invertebrates, and small vertebrates, i.e., smaller than the animal itself. Also unlike eulipotyphlans, there is no caching behavior observed, with all food being consumed immediately. Testing the brachial gland exudate on arthropods did not confirm deleterious effects on this type of prey. Predator defense is somewhat unlikely, since the predators are diverse and the loris-predator encounters are rare. However, when testing BGE-saliva mixes on olfactory-oriented predators (leopards, clouded leopards, tigers, sun bears, common palm civets, and binturongs), these were repelled by the venom. In the field, it has been observed that Javan slow lorises move close to palm civets and leopard cats. Visually oriented predators reacted differently when faced with loris venom. Eagles (*Spizaetus*, *Spilornis*) show inconclusive behavior, while orangutans actually eagerly consumed the venom-containing swabs. These visual predators (along with pythons) are known to prey on lorises (Hagey et al. 2007). There is growing evidence that the secretion may act as an antiparasitic, since lorises are conspicuously low in ectoparasites, being slightly more affected in the rainy season. Tests on leeches and tick-related models revealed that the BGE-saliva mixture is able to kill them.

The venom may have a role in intraspecific competition (as observed for solenodons and platypuses). Loris-on-loris bites are severe, affecting large areas with loss of fur, prolonged edema, and are slow-healing (often life-threatening) (Hagey et al. 2007). The chemical complexity of BGE, associated with grooming behaviors, point to the substance being used as a signaling device. The main target, however, may not be predators but the lorises themselves. Aside from fending off some predators, the secretion may alert other lorises about the predator's presence. Since the venom shows species specificity, this may reinforce a communication role. Taking its high similarity to Fel d 1, the major cat allergen, structural modeling suggest that the BGE protein may act as a box (Fig. 9), being able to close its lid on

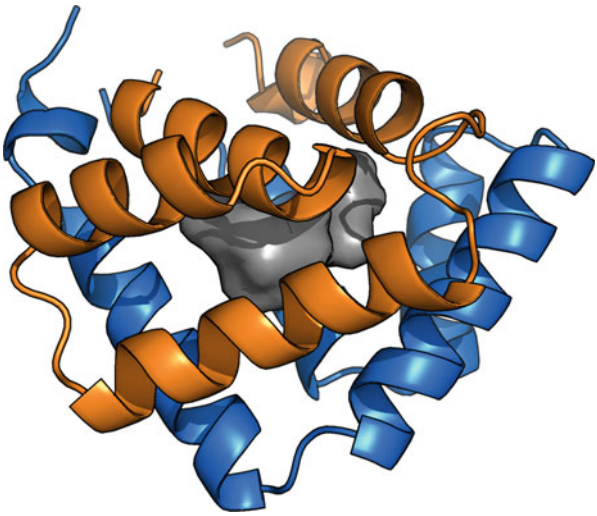

Fig. 9 Fel d 1, a working model for BGE protein. α-chain in *blue*, β-chain in *orange*. The internal cavity, proposed to act as a box, is shown as a *grey* volume (PDB ID 2EJN, Kaiser et al. 2007) (Author's own artwork)

signaling molecules from saliva or BGE. This entrapping and delivery system helped propose the physiological role of Fel d 1 in big and small cats (Ligabue-Braun et al. 2015). It is interesting to note that chimpanzee and human genomes harbor remnants of the BGE protein in the form of pseudogenes, indicating a putative venomous past for apes (Hagey et al. 2007) or a long lost redundant uteroglobin.

Nekaris et al. (2013) proposed an elegant hypothesis for the role of venom in lorises as part of a much broader Müllerian mimicry system. In this proposal, the use of venom to repel olfactory-orientated predators is combined with other features, such as extra vertebra in the spine that allow serpentine movements, aggressive snake-like vocalizations, and long dark dorsal stripes and dark ocular circles, constituting a mimic of cobras (*Naja* spp.). Despite its multipurpose role (including intraspecific competition and parasite defense), the evolution of venom may have been an adaptive strategy against predators when combined with other features, arising in the Miocene, when slow lorises and cobras migrated through Southeast Asia.

Recently, Harris and Arbuckle (2016) used large datasets with comparative phylogenetic methods to inspect patterns of venom and poison evolution in birds, amphibians, reptiles, and mammals. They found that venom biosynthesis evolution is much less dynamic than that of toxin sequestration from the diet. Apart from amphibians, the remaining tetrapods show an association between the evolution of toxins and higher diversification rates. Furthermore, the work found that mammals and reptiles evolve under a similar regime regarding their toxicity/venomousness, with gains and losses of toxicity sparsely and infrequently distributed across the phylogeny. Interestingly, mammals form the only tetrapod lineage in which venom is used for intraspecific competition. Harris and Arbuckle (2016) speculate that, due to frequent social interactions in mammals (compared to other groups), they may be under higher selection pressure to use venom in social situations. To support this,

the authors argue that such behavior is observed in some eusocial hymenopteran insects.

Folinsbee (2013) summarized the main (nonmutually exclusive) hypotheses to why venom is rare among extant mammals. Venom may not be adaptive in mammals (i.e., there is no need for it); the production of venom may be constrained by some biological factor; there are high costs associated with production and maintenance of venomous secretions; and venom may be adaptive only in a narrow range of morphologies. Regarding the need for venom, the greater size and masticatory adaptions acquired by mammals may have supplanted the (putative) ancestral venomousness. Still, it is unclear, at least for the diverse Eulipotyphla order (452 extant members), what proportion is really venomous. There is an abundance of untested mammals in this order to be evaluated prior to considering them nonvenomous. Studying them comparatively may confirm (or disprove) multiple origins for venom in these animals. The cost of producing venoms also needs to be evaluated in mammals. Snakes and arachnids carefully measure the amount of venom deployed in each attack, with pitvipers and death adders increasing significantly their metabolic rates when synthesizing venom (Folinsbee 2013; Morgenstern and King 2013). There is still no study regarding venom production costs in sister mammalian taxa that would clarify the energetic costs of using venom for predation. If it is not costly, another constraint must be in place. A likely explanation is that the highly specialized mammalian teeth, intensively used for oral processing of food, may hinder their modification into venom delivering tools, making venom less adaptive. It is possible that a combination of factors makes venom adaptive only for a specific phenotype, namely, small body size with high metabolism (Folinsbee 2013). As becomes clear when one inspects each mammalian order's venom, its uses, and injecting apparatus, there is no homology present (at least, none that can be ascertained without major concessions). Venomousness seems to have independently arisen at least four times among mammals, once in each of the four orders presented in this chapter. Explaining the scarcity of venomous mammals may take into account specificities of each mammalian order.

Anthropocentric Biases: Research Limitations, Exciting Applications

The study of venoms in general suffers from a human-centered perspective. This is understandable, since humans are the ones doing research, basic science is expensive, and resources are scarce. However, this should be avoided as soon as perceived as an obstacle to fully understand venoms and toxinology as a whole. The study of venomous mammals also have these caveats.

Most of the available data reflects venom effects on humans, pets, farm, or experimental animals. Venom effects, however, are context- and taxon-specific. For this reason, using lethal dosage evaluation (LD_{50}), for instance, is problematic, since it is normally based on a single species, ignoring that different species respond

differently to the same compound (Brodie 2009). Only recently there has been an effort to understand the physioecological role of venom in the animals' life history.

Despite mammalian venoms reflecting such bias, there are indeed tests on animals that are more strongly related to the mammals' ecology. For instance, roaches and crickets were tested with Eulipotyphla, in an attempt to mimic their invertebrate diet, and lorises had their venom tested on spiders, maggots, ants, fleas, and caterpillars (Grow et al. 2015).

Much information regarding venom use by mammals originated from observational studies. Still, there is room for more assessments aiming to understand venom use by these animals in their natural habitat. Since the notion of venomousness as a mammalian trait is just beginning to be accepted by the wider zoology community, there are many gaps still waiting to be bridged in respect to these animals. The vast majority of venomous mammals are difficult to maintain in captivity, and even when this is not a limiting feature, the amount of venom is too small to allow in-depth research without straining individual animals or requiring large numbers of individuals. Genomic techniques are rising as possible answer to this conundrum.

Toxic proteins from mammals have served as models to understand mammalian evolution, as well as providing interesting prototypes for new drugs. Anticoagulants from vampire bat saliva have been proposed as promising treatments for myocardial infarction, pulmonary thromboembolism, and stroke, since a key aspect of these events is to keep blood unclotted. BGE protein from lorises may help to assess allergy-related issues in humans, considering its high similarity to cat allergens. In its turn, platypus venom may aid in the study of pain perception and as a model to design novel pain relievers, particularly interesting when one targets long lasting, treatment-unresponsive pain. Isomerases from Monotremata venom may offer tools to develop degradation-resistant peptides with medical application. Other, less studied, toxins from this venom may even work as scaffolds for antineoplastic drugs (Ligabue-Braun et al. 2012, 2015; Rode-Margono and Nekaris 2015; Whittington and Belov 2016). The field of venomics is growing together with toxin-based drug discovery (Calvete 2009). Mammalian venoms are thus rich sources of novel frameworks for drug development.

Conclusion and Future Directions

Mammalian venoms prompt a need to revise the definitions of venomous and poisonous animals. In the traditional definition, venoms are produced and stored in specialized structures (glands), associated with delivery devices, forming the envenomation apparatus with which the venoms is delivered directly to the recipient's body. Thus, venomous animals are actively toxic. Poisons may be available in specialized structures in the toxic animal but lack any special mechanism of delivery. The recipient animal must eat (or at least be in direct contact with) the poisonous animals to be affected. This is considered passive toxicity. There are obvious flaws with these definitions. For instance, the spitting cobra would be considered poisonous and not venomous, when venom is delivered by squirting and not directly onto

the prey. Likewise, the feeding secretions from hematophagous animals are not universally considered as venom, even though they satisfy all requirements to be. It is the fact that these animals depend on the survival of the food source for continuous supply of nutrients that may divert them from traditional definitions, despite their venoms being capable of facilitating feeding by disruption of normal physiological processes of the prey.

Eulipotyphlans and platypuses satisfy the criteria to be taken as venomous. Vampire bats are considered venomous only if hematophagy is enrolled along with traditional venom uses (Fry et al. 2009). Lorises clearly do not satisfy the criteria, since their venom-producing organ is not directly connected to the injury-inflicting and toxin-delivery apparatus. Still, one can no longer argue that these animals cannot act venomously.

Chemical defenses (passive toxicity) are also present in mammals. Pangolins, skunks, the greater long-nosed armadillo (*Dasypus kappleri*), and the striped polecat (*Ictonyx striatus*) emit noxious substances to fend off predators. This form of chemical defense would allow these mammals to be considered poisonous. In the most striking example of chemical defense in mammals so far, the African crested rat (*Lophiomys imhausi*) is able to sequester toxic substances from plants and accumulate them in their manes, forming a protective mantle. These cases have been considered "arguably venomous" (Rode-Margono and Nekaris 2015). Considering that examples of chemical defense in other animal groups are ample (amphibians), understudied (marine turtles) and still being elucidated (birds) (Ligabue-Braun and Carlini 2015), it is possible that mammalian poisons may be much more widespread than mammalian venoms. Hopefully this chapter, along with recent literature, will be able to include mammals in the "hall of venomous animals" from the perspective of both toxinologists and the general public.

Cross-References

- ▶ A Critique of the Toxicoferan Hypothesis
- ▶ Evolution of Resistance to Toxins in Prey
- ▶ Evolutionary Context of Venom in Animals
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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Part III

Evolution of Venom Delivery Systems

Evolution, Morphology, and Development of the Centipede Venom System

13

Michel M. Dugon

Abstract

With approximately 3,500 species distributed across five extant orders, centipedes (class Chilopoda) make the second most speciose class among the subphylum Myriapoda. The most conspicuous synapomorphic character of centipedes is certainly the modification of the first pair of legs into powerful venomous forceps (the forcipules). The venom gland encased in each forcipule produces a potent cocktail of paralytic toxins delivered into prey and opponents via a cuticular duct which opens on the subterminal part of the apical claw. It has been hypothesized that this modification, unique in the animal world, results from the folding of the outer cuticle of the walking legs and the transformation of related subepidermal gland units into venom-producing cells as an adaptation to a new terrestrial predatory niche over 430 million years ago, thus making centipedes one of the most ancient known clade of terrestrial venomous organisms. However, despite their global distribution, synanthropic habits, and reputation for inflicting painful stings, little is known about centipedes and their venom system. This chapter reviews the current knowledge on the development, the evolutionary trajectory, the anatomy, the physiology, and the predatory ecology of centipedes, with a strong emphasis on the forcipular apparatus.

Keywords

Centipedes • Forcipules • Maxillipeds • Venom system • Chilopoda

M.M. Dugon (✉)
Discipline of Zoology – School of Natural Sciences, National University of Ireland Galway, Galway, Ireland
e-mail: micheldugon@hotmail.com; michel.dugon@nuigalway.ie

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_1

Contents

Introduction

> For because Minos cohabited with many women, Pasiphae bewitched him, and whenever he took another woman to his bed, he discharged scorpions, serpents and centipedes at her joints, and so the women perished.
>
> Pseudo-Apollodorus, *Bibliotheca* 3.15.1

Feared and revered for their venomous sting and feisty nature, centipedes have been the center of several myths and tales around the world. In ancient Egypt, the Goddess Sepa had the shape of a centipede and was implored by prayers to cure snake bites and increase fertility. Her cult developed particularly in Heliopolis and later merged with the cult of Osiris. Several thousand kilometers away, the Chinese centipede (Wu-Gong) was linked to tales of dragons and thunder as a symbol of strength and power. In Indonesia, "batu mustika lipan" (allegedly centipede bezoars, sometimes said to be worn as a crown by the animal) are said to hold powerful mystical attributes. In the state of Seremban in Peninsular Malaysia, a temple has been built on the site of the Wu-Gong San, for devotees to ask favors of the spirit of a giant centipede roaming the local hills.

In Western literature, centipedes are probably first presented as venomous creatures by Pseudo-Apollodorus in the *Bibliotheca*, a text attributed to the first or second century AD. However, the myth of Minos' infidelities to his wife Pasiphae is much older and brings us back deep into the mythical roots of the Hellenist Era and the Minoan culture.

One and half millennia later, Leeuwenhoek (1719) was the first European scientist to write about the venom claws of centipedes. In a surprisingly successful attempt, Leeuwenhoek provides a rather precise account of the external morphology of the claws, taking much care in locating the duct opening on the subterminal end of the claw from which venom is secreted. Leeuwenhoek's description appears under the term "Millepaeda." The distinction between millipedes and centipedes was to be clarified by Linnaeus four decades later (1758), with the description (still valid to this day) of a large Mediterranean centipede, giving it the appropriate name of "biting centipede" (*Scolopendra morsitans*).

Despite detailed account of the effects of a centipede bite and accurate morphological descriptions (e.g., Newport 1844), the existence of a venom apparatus linked to the claws was debated until the ultrastructural description of the venom gland by MacLeod (1878) and Duboscq (1898). The two latter authors are recognized as the pioneers in the study of the venom claws, and both their publications are landmarks and starting points for all those interested in the study of the venom system of centipedes.

This chapter explores some of the current knowledge on centipedes with a strong emphasis on the evolution, the development, the morphology, and the functionality of their venom system, the *forcipular apparatus*.

Phylogeny and Diversity

Along with millipedes (Diplopoda), pauropods (Pauropoda), and symphylans (Symphyla), centipedes (class Chilopoda) form the subphylum Myriapoda. Myriapods are terrestrial, mainly ground dwelling, animals present on all continents except Antarctica. Myriapods are easily recognizable by their body divided in two tagmata (head + abdomen and absence of thorax), and their multisegmented trunk bearing one or two pairs of legs per segment. The number of trunk segments and related appendages is highly variable (12–191 trunk segments). The head always bears four pairs of cephalic appendages (antennae, mandibles, first maxillae, and second maxillae). The gas-exchange system is composed of a tight network of tracheae and spiracles. When present, the eyes are usually composed of a variable number of simple ocelli, with at least one order of centipedes possessing compound eyes.

However, while millipedes, pauropods, and symphylans feed almost entirely on plant and decaying matter, centipedes are fundamentally predators, using the highly modified legs of the post-cephalic trunk segment to subdue their prey before devouring them. This is arguably the most conspicuous feature of the clade and the only known example in the animal kingdom of the modification of legs into venom-injecting appendages.

Morphologically, centipedes are generally characterized by a long and slender, dorsoventrally compressed body protected by rigid cuticular plates (ventral sternites and dorsal tergites) separated by flexible membranes. Upon maturation, centipedes bear 15–191 pairs of legs, as one pair per trunk segment (Minelli et al. 2000). Most specimens from temperate areas measure from 1 to 10 cm, with larger tropical species attaining lengths in excess of 30 cm (Lewis 1981).

With approximately 3,500 described species, centipedes make the second most speciose class among the subphylum Myriapoda. The class Chilopoda is further divided into two subclasses containing a total of five extant orders and one extinct order. The subclass Notostigmophora contains the archaic order Scutigeromorpha, while the subclass Pleurostigmophora contains the orders Lithobiomorpha, Craterostigmomorpha, Scolopendromorpha, Geophilomorpha, and the extinct Devonobiomorpha.

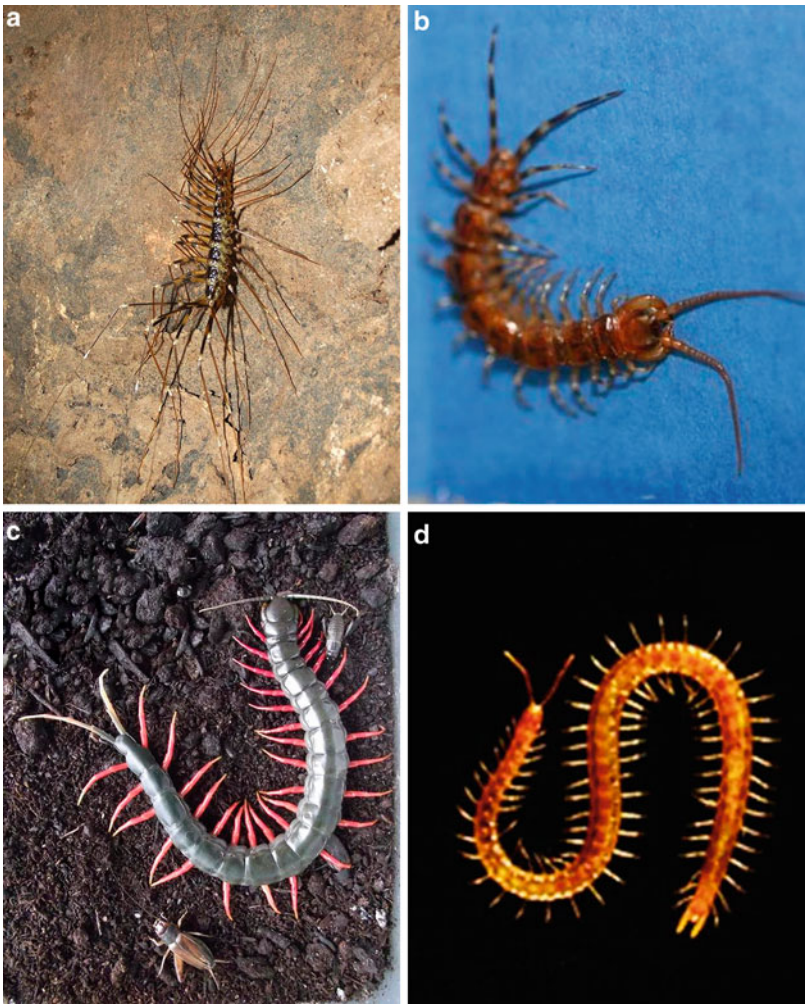

Fig. 1 Representatives of the four main extant orders of centipedes: (**a**) *Thereuopoda longicornis* (Scutigeromorpha), (**b**) *Lithobius variegatus* (Lithobiomorpha), (**c**) *Scolopendra subspinipes* (Scolopendromorpha), and (**d**) *Strigamia maritima* (Geophilomorpha)

– The order Scutigeromorpha (house and cave centipedes, Fig. 1a) is a small (c. 200 species) order of centipedes distributed mainly in the tropical and subtropical belt, with a cosmopolitan synanthropic species (the house centipede *Scutigera coleoptrata*). The body is proportionally short and cylindrical, supported by long and thin appendages. Development is anamorphic: the hatching larva has four pairs of legs, and new segments are added in subsequent molts to reach 15 pairs of legs upon maturity. Scutigeromorph centipedes display many features differentiating them from other centipedes, such as dorsal spiracles (as opposed to lateral spiracles in other orders), compound eyes (as opposed to simple ocelli or

complete absence of eyes), and leg-like, stiletto-shaped venom claws (as opposed to stout forceps-like claws).
- Members of the order Lithobiomorpha (Fig. 1b, stone centipedes, c. 1,150 species) are small (8–40 mm), dorsoventrally compressed centipedes distributed worldwide, with some notable synanthropic species (e.g., *Lithobius forficatus*). Development is anamorphic with hatchlings bearing four pairs of legs. Mature specimens have 15 pairs of legs.
- The order Craterostigmomorpha comprises only two small to medium (c. 50 mm) species confined to Tasmania and New Zealand. Egg clutches are guarded by the mother. Hatchings possess 12 pairs of legs and add the remaining three pairs in a single molt.
- Centipedes from the order Scolopendromorpha ("giant" centipedes, c. 700 species, Fig. 1c) are distributed worldwide and range in size from 10 to 300 mm. Large species (over 150 mm) are mainly distributed within the tropical belt. Development is epimorphic: the number of legs (normally 21 or 23 pairs, with a single species having 39–43 pairs) is family-specific and remains fixed throughout lifetime. Simple eyes (ocelli) can be present or absent. This is the only order of centipedes known to inflict medically significant stings.
- Geophilomorpha (earth centipedes, Fig. 1d) is the most speciose order, comprising small to long thread-like burrowing species and is distributed worldwide. Development is epimorphic with extended maternal care. The number of leg-bearing segments (LBS) varies greatly within and between species (27–191 LBS).
- Devonobiomorpha is an order created to accommodate the Devonian centipede *Devonobius delta* originally described by Shear and Bonamo (1988) from the Middle Devonian sediments of Gilboa (USA).

The Forcipular Apparatus

External Structure

The venom claw (or forcipular) segment is a modification of the first post-cephalic, leg-bearing trunk segment. Modifications involve the whole internal and external structure of both the segment and the pair of appendages. It has been hypothesized that this modification, unique in the animal world, results from the folding of the outer cuticle of the walking legs and the transformation of related subepidermal gland units into venom-producing cells as an adaptation to a new terrestrial predatory niche over 430 million years ago (Dugon and Arthur 2012a).

The segment's outer cuticle is heavily sclerotized. On the dorsal aspect, the head capsule largely overlaps with the forcipular dorsal plate (tergite), except in the order Scutigeromorpha. On the ventral aspect, the two sternal plates are fused into a strong sternite (Fig. 2a), except in the order Scutigeromorpha where they remain separated by a thin membrane providing flexibility to the sternal shield (Fig. 2b). In all other centipedes, the sternite curves laterally and protects the anchorage points of the

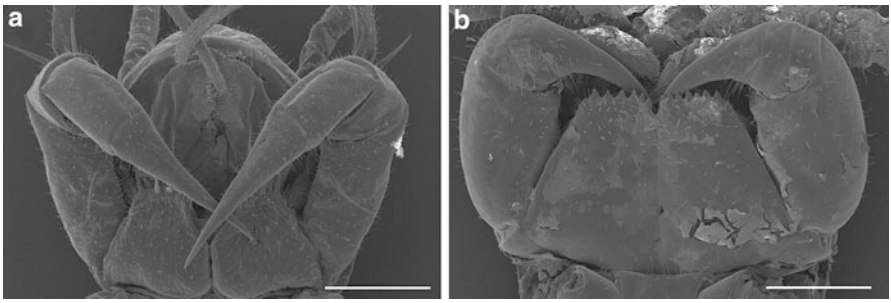

Fig. 2 Ventral aspects of the forcipular apparatus of (**a**) the scutigeromorph *Scutigera coleoptrata* and (**b**) the lithobiomorph *Lithobius forficatus*. SEM micrographs. Scale = 1 mm

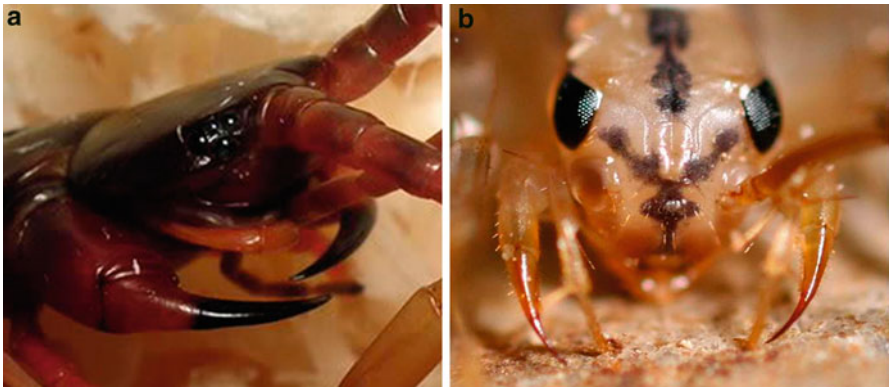

Fig. 3 Details of the head capsule and forcipules of the scolopendromorph *Scolopendra hainanum* (**a**) and the scutigeromorph *Scutigera coleoptrata* (**b**)

forcipules located on the sides of the segment. The sternal plates usually bear notches (replaced by long bristles in Scutigeromorpha) projecting anteriorly under the mouth.

Each forcipule is composed of four segments. From proximal to distal, these four segments are (1) the trochantero-prefemur (resulting from the fusion of the trochanter and the prefemur), (2) the femur, (3) the tibia, and (4) the tarsungulum. The tarsungulum is composed of two fused segments, the tarsus and the apical claw (Dugon et al. 2012a). Each forcipules can move independently from the other. A set of strong condyles and muscles provide mobility (although limited) to the trochantero-prefemur in the three plans; the femur and the tibia are limited to lateral movements (Pleurostigmophora) or vertical movements (Notostigmophora) (Fig. 3).

Careful examination of the tarsungulum reveals the presence of a small opening (meatus) from which venom is secreted on the outer subterminal part of the apical claw. The diameter of the meatus varies greatly from species to species and seems positively correlated to the overall size of the specimen, ranging from 2 μm in

smaller species of geophilomorphs to over 50 μm in large scolopendromorphs. The meatus is prolonged by a groove extending distally toward the tip of the claw which may increase the effectiveness of venom delivery (Dugon et al. 2012a).

The outer cuticle of the forcipules is covered with a tight network of microscopic sensillae involved in the reception of mechanical and chemical stimuli. The apical claw bears three types of short sensilla coeloconica emerging from depressions in the cuticle. Sensilla coeloconica bear a small pore on their apex and their density increase on the most distal part of the apical claw, thus suggesting a chemoreceptive role. The more proximal articles bear various trichomes embedded in large socket, presumably fulfilling mechanoreceptive functions (Dugon et al. 2012a; Ernst and Rosenberg 2003). Scutigeromorpha possess two rows of club-shaped trichomes on the inner curvature of the tarsus which are involved in the preening of the antennae and legs (Rosenberg et al. 2004).

The Venom Gland

Location and Shape of the Venom Gland

The venom gland encased in each forcipule produces a potent cocktail of paralytic toxins delivered into the prey via a cuticular duct which opens on the subterminal part of the apical claw (Undheim and King 2011). The venom duct is an invagination of the exoskeleton penetrating the mass of the forcipule (Dugon and Arthur 2012a).

The size and location of the venom glands within the forcipules are very variable between and within orders. In the anamorphic species (scutigeromorphs and lithobiomorphs), the venom gland usually extends distally in the tarsungulum and reaches proximally down to the trochantero-prefemur (Undheim and King 2011). In scolopendromorphs, the length of the venom glands differs between families, but it seems always contained within the forcipular segment. Variations are greatest in geophilomorphs. Among the members of the family Schendylidae, the venom gland is contained in the tarsungulum. In the Dignathodontidae *Henia vesuviana*, the venom gland is located between the 12th and 18th trunk segments (Duboscq 1898).

Ultrastructure and Function of the Venom Gland

The venom gland is composed of a glandular epithelium arranged radially around the proximal porous part of the venom duct (the calyx) (Fig. 4). During injection, the venom stored in the glandular epithelium is released into the lumen of the duct via small pores on the calyx. Each pore measures 1–2 μm in diameter and connects a single-venom secretory unit to the lumen of the venom duct. In Geophilomorpha, the short, bulbous calyx bears pores on its entire circumference. In the other orders, pores are not present on the side of the calyx closest to the cuticle. The distal part of the duct is smooth, without perforation, and follows the outer curvature of the forcipule to open into the meatus on the outer subterminal part of the apical claw (Dugon et al. 2012a).

The venom gland is surrounded by an epithelial basal lamina attached on the outer-lateral side of the venom duct. In Scolopendromorpha and Scutigeromorpha,

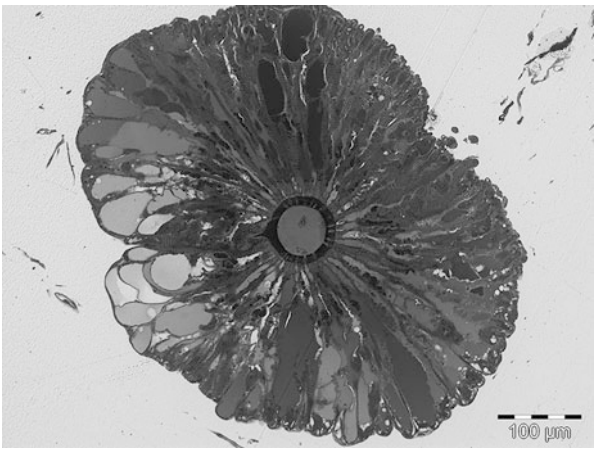

Fig. 4 Cross section through the venom gland of *Scolopendra subspinipes mutilans*. The venom secretory units are arranged around the porous proximal part of the cuticular duct (calyx)

peripheral muscles surround the glandular epithelium. Additionally, centripetal muscles attached to the calyx and the peripheral muscles run between the secretory units of the venom gland, thus suggesting that venom release is under muscular control (Antoniazzi et al. 2009; Dugon and Arthur 2012a).

The glandular epithelium is composed of hundreds of secretory units, each comprising four cells: (1) a *proximal canal cell*, (2) a *distal canal cell*, (3) an *intermediary cell*, and (4) a *secretory cell* (Rosenberg and Hilken 2006).

The *proximal canal cell* forms the clove-shaped valve, occupying the cuticular atrium and the more cuticular pads forming the cap of the pore. It is thought to control the secretion of venom into the lumen of the duct. The *distal canal cell* encloses the cuticular atrium opening on the main lumen of the calyx. The long and thin *intermediary cell* encloses a large extracellular space filled with the solubilized venom produced under the form of granules by the *secretory cell*. The granules produced by the secretory cell are thought to be released in the extracellular space via exocytosis.

The venom stored in the extracellular vacuoles is squeezed through the cuticular pads of the proximal canal cell, enters the atrium of the cuticular pore formed by the proximal canal cell, and is released into the main lumen of the venom duct. The clove-shaped atrial valve stops the venom from rushing back into the glandular structure and may also act as barrier against bacterial infections (Dass and Jangi 1978).

The observations conducted on scolopendromorphs by early authors (e.g., Duboscq 1898; Barth 1967) led them to believe that the venom gland was holocrine in nature. However, more recent studies (Menez et al. 1990; Antoniazzi et al. 2009) did not find any evidence of cell degeneration. It is likely that the extracellular venom vacuoles were misinterpreted as degenerated cells (because of the absence of organelles). The venom secretion may in fact be merocrine (Undheim and King 2011).

The venom production cycle in centipedes has not been extensively investigated. From various TEM investigations in scolopendromorphs, it seems that electron-dense

granules are formed in the secretory cell (Dass and Jangi 1978; Menez et al. 1990; Antoniazzi et al. 2009). The occurrence of these granules is preceded by the multiplication of large strains of rough endoplasmic reticulum. The granules show sometimes lighter rod-like inclusions, which have been interpreted as a possible sign of solubilization before exocytosis (Menez et al. 1990).

Venom Regeneration

Venom regeneration has been investigated in the common desert centipede *Scolopendra polymorpha* (Cooper et al. 2014) and to a lesser extent in the Chinese red-head centipede *Scolopendra subspinipes mutilans* (Dugon and Arthur 2012b).

In captivity, the predatory behavior of *Scolopendra subspinipes mutilans* is significantly altered, following venom extraction by electrostimulation. Usual prey (i.e., small crickets and larger migratory locusts) are refused in the hours following complete depletion of the venom gland. Crickets are accepted again 24 h after venom extraction and locusts 48 h after venom extraction (Dugon and Arthur 2012b).

Cooper et al. (2014) found that venom regeneration in the common desert centipede *Scolopendra polymorpha* occurs most rapidly in the first 48 h following complete depletion (65–86% regeneration) and then plateau for several weeks. While the volume of venom is rapidly restored after secretion, protein mass remains low and protein compounds appear to be produced asynchronously. Near-full regeneration occurs only several months after extraction, although this might have been due to structural damages to the venom gland during the extraction procedure. The long regeneration cycle would suggest a period of latency during which centipedes may be vulnerable to predators; however, it is unlikely that centipedes use the full content of their venom gland during a predation episode.

Ontogeny of Centipedes with Reference to the Forcipular Apparatus

In general, centipedes are sexually reproducing arthropods, although some geographically restricted populations of some geophilomorph and lithobiomorph species are suspected to be parthenogenetic (Enghoff 1975; Bonato et al. 2005). The life cycle of centipedes is relatively slow when compared to most other arthropods. For many taxa, life expectancy is estimated to be at least 2–6 years (Minelli and Sombke 2011) but it may actually be longer for some large scolopendromorphs. Sexual maturity is reached after approximately 1 year for *Lithobius erythrocephalus* (Voigtländer 2006) and after 2 years for *Strigamia maritima* (Lewis 1981). The scolopendromorph *Rhysida nuda* matures within 2 years (Lewis 1981). The ontogeny of centipedes is strongly influenced by the presence (Craterostigmomorpha, Scolopendromorpha, and Geophilomorpha) or absence (Scutigeromorpha and Lithobiomorpha) of maternal care.

Reproduction, Egg Laying, and Maternal Care

Usually, a courting ritual takes place, involving a careful and long (sometimes several hours) approach, defensive postures, and tapping with the legs and/or antennae on the extremities of the partner (cf. Lewis 1981 for a review). In lithobiomorphs, scolopendromorphs, and geophilomorphs, the male produces a web on which the sperm is deposited before being collected by the female. Male scutigeromorphs, lithobiomorphs, and scolopendromorphs produce spermatophores, while some geophilomorphs appear to deposit an uncased sperm droplet (Lewis 1981). According to Minelli (2011) females are unlikely to receive sperm more than once a year and most probably are impregnated only once in their lifetime. A female captive specimen of *Ethmostigmus trigonopodus* (Scolopendromorpha) mated only once produced two clutches 144 days apart (Iorio and Ythier 2007).

Scutigeromorpha, Lithobiomorpha, and Craterostigmomorpha present an anamorphic development. The hatchlings are fully mobile and leave the egg with an incomplete number of trunk segments. The remaining segments develop after successive molts. The eggs are laid in a small cluster or individually, covered with soil, and then abandoned by the mother. Scolopendromorphs and geophilomorphs are incapable of movements when hatching, but possess already their final number of trunk segments (epimorphic development). In these two orders plus Craterostigmomorpha, the mother takes care of the brood for several weeks or months, until the young are sufficiently developed to move and hunt on their own.

Development of the Venom Apparatus

Little is known about the development of the venom system during embryogenesis. At the early stages of development, the forcipular segment is morphologically similar to other trunk segments. Enlargement and repositioning of the forcipules become noticeable only in mid to late pre-hatching developmental stages (Fig. 5). However, *Hox* gene expression patterns in the forcipules of the geophilomorph *Strigamia maritima* reveal a forcipule-specific *Hox* expression (Hayden and Arthur 2013) in earlier germ-band developmental stages.

The forcipular appendages and the venom gland are not functional in newly hatched centipedes. In geophilomorphs and scolopendromorphs, the forcipules of freshly hatched specimens are somewhat enlarged compared to the leg buds and held perpendicular to the body. The forcipules are blunt and soft and lack articulations and sclerotization. The venom gland and the venom duct are absent. The forcipules of hatching lithobiomorphs and scutigeromorphs appear to be segmented. Although there is no maternal care, the developmental stage immediately following hatching is believed to be nonfeeding, indicating that the venom system is still nonfunctional at this stage (Dugon et al. 2012b).

The development of the venom glands of the Chinese centipede *Scolopendra subspinipes mutilans* has been reported by Dugon and Arthur (2012b). In the second postembryonic stage, the forcipules are still held perpendicular to the trunk, and the

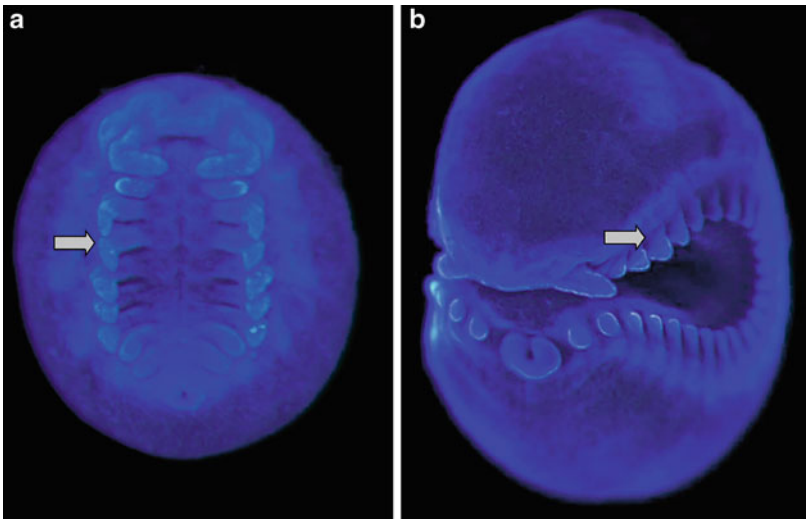

Fig. 5 (**a**) Embryo of the scutigeromorph *Scutigera coleoptrata* (anamorphic development) at mid-development. (**b**) Embryo of the scolopendromorph *Scolopendra subspinipes mutilans* (epimorphic development) at the hatching stage. The *arrows* point to the forcipular buds

venom gland is absent. At the third postembryonic stage (about 35 days after hatching), a cuticular fold on the dorsolateral aspect of the apical claw is shaping into the venom duct. At the same time, the glandular epithelium emerges from the internal wall of this fold and grows posteriorly toward the trochantero-prefemur. The full extension of the venom gland is reached within 4–5 days. Although most of the venom duct is still embedded in the dorsolateral cuticle of the claw, the forcipules and the internal venom apparatus appear to be fully functional at the adolescence stage, approximately 7–8 weeks after hatching. In the adult *Scolopendra subspinipes mutilans*, an indent in the cuticle is visible on the dorsal aspect of the tarsungulum, where the duct sank into the mass of the forcipule during development. On the basis of these developmental observations and the evolutionary trajectory of the venom claw morphology from a *Scutigera*-like ancestor some 420 million years ago, it has been suggested that the development of the venom apparatus of *Scolopendra* recapitulates, at least partially, the evolution of the apparatus (Dugon et al. 2012b).

Fossil Records of Centipedes with Special Reference to the Forcipular Apparatus

The fossil record of myriapods is sparse and very incomplete. However, several major discoveries have been made in the last three decades, providing an insight into the deep phylogenetic nodes of the subphylum. Most of these discoveries have been compiled and discussed in four major reviews (Almond 1985; Shear 1997; Shear and

Edgecombe 2010; Edgecombe 2011) which, when read in chronological order, offer an interesting view of the large progress made in reconstructing the evolutionary history of the myriapods.

The absence of calcium carbonate in the cuticle of chilopods is the main reason for the low number of fossilized remains (Shear and Edgecombe 2010). No stem-group myriapod fossils have been definitely assigned so far. The earliest confirmed myriapod records have been dated to the early and mid-Silurian and described as primitive diplopods (Almond 1985). However, speculations on a Cambrian-Ordovician myriapod have not been dismissed and are sometimes debated (Shear 1997; Wilson 2006). Using the fossil record, combined with morphological features and molecular data, Murienne et al. (2010) were the first to propose a chronogram for the centipede tree of life.

The oldest confirmed chilopod fossils belong to the genus *Crussolum*, a scutigeromorph recovered from the Scottish Rhynie Chert, a hot-spring vent location dated from the Early Devonian (407 MYA) (Anderson and Trewin 2003) and from the Gilboa site in New York State (Shear et al. 1998). The Scottish specimen presents perfectly preserved forcipular coxal plates and partially preserved forcipules. *Crussolum* has forcipular attributes that are typical of the extant Scutigeromorpha: separated coxal plates, insertion point of the elongated trochantero-prefemur, long setae on the distal end of the coxal plates, and absence on the posterior end of the coxae of apodemal insertions into the first trunk segment.

Later Scutigeromorpha fossils were identified in the Mazon Creek deposits of Illinois (Upper Carboniferous), USA (Shear and Edgecombe 2010), and in the Crato Formation of Northeast Brazil (*Fulmenocursor tenax* Wilson 2001). *Fulmenocursor tenax* (Lower Cretaceous) has been assigned to the family Scutigeridae on the base of the possession of antennomeres that are longer than wide.

Devonobius delta (Chilopoda: Devonobiomorpha: Devonobiidae), a member of an extinct order of centipede from the Middle Devonian, has been described from the Gilboa deposits (Shear and Bonamo 1988). Upon examination, *Devonobius* was placed between the Craterostigmomorpha and the Epimorpha (Scolopendromorpha + Geophilomorpha). This central position is interesting, as some transitional morphological features may be present in the specimens.

The forcipules of the *Devonobius* specimen are relatively well conserved. The coxosternite is in one block (while all the previous centipede fossils presented two separated coxal plates as in Scutigeromorpha), with a ridge visible where the fusion between the two coxae occurred. The coxal tooth plates project forward in a manner similar to some Scolopendromorpha. The forcipules are long and project far distally. The general shape and organization are very close to that of *Craterostigmus tasmanianus*, a parallel noted by Shear and Edgecombe (2010). The forcipules possess a long trochantero-prefemur and a long tarsungulum, but a much reduced femur and tibia. However, and unlike the Epimorpha, the trochantero-prefemur and the tarsus do not share an articulation point. Each forcipular article forms a complete ring around the limb.

Scolopendromorpha representatives have been found in the Crato Formation of Brazil and the Mazon Creek of Illinois, with three named species: *Mazoscolopendra*

richardsoni (Mundel 1979) *Cratoraricus oberlii* (Wilson 2003), and *Velocipede betimari* (Martill and Barker 1998). The forcipules of the four specimens present the typical form of scolopendromorph forcipules: enlarged trochantero-prefemur and reduced and incomplete femur and tibia, with a stout tarsungulum. The tarsungulum and trochantero-prefemur touch each other on the external lateral part of the forcipule, a synapomorphic character of the modern epimorphs.

According to Shear and Edgecombe (2010), the fossil records of both Lithobiomorpha and Geophilomorpha are very limited. Regarding Lithobiomorpha representatives, the authors mention that the "fossil record is confined to the Cenozoic, with several taxa having been named from Baltic amber, though none has received modern study." Considering the rather "young" age of such specimens, it is unlikely that the forcipular system looked any different from the one existing today in Lithobiomorpha.

As for the Geophilomorpha, the earliest fossil is a single specimen from the Upper Jurassic, *Eogeophilus jurassicus* (Schweigert and Dietl 1997). The minute size of the specimen and the rather bad preservation make the photographic material that is available difficult to interpret in terms of forcipule shape.

Another single specimen of geophilomorph from French amber, *Buziniphilus antiquus* (Edgecombe et al. 2009), was dated from the early Cenomanian (Upper Cretaceous, 93–100 MY). This specimen is interesting for the very clear view it offers on an undamaged forcipule. It appears that the trochantero-prefemur and the tarsungulum are linked by a joint as in all known living Geophilomorpha. The pleuritis seems very developed. Interestingly, the embedment in amber permits distinguishing the venom duct and the porous proximal extremity, the calyx. The calyx appears very developed and large in comparison to all of the living species I have examined. However, the calyx is mostly confined to the femur/tarsungulum part of the claw, a common occurrence in living geophilomorphs. The specimen was placed into the suborder Adesmata and belongs to either of the families Geophilidae or Schendylidae.

Predatory Behavior and Prey Choice

Although all centipedes are thought to be opportunistic predators rather than specialist feeders, their predatory behavior is strongly correlated to their morphotype and the specific ecological niche they occupy (Voigtländer 2011). Three main morphotypes were identified by Manton (1977): (1) running type (Scutigeromorpha and Lithobiomorpha), (2) burrowing type (Geophilomorpha), and (3) intermediate type (Scolopendromorpha and Craterostigmomorpha).

In all five orders the anterior legs and forcipules are all involved in holding and subduing prey. In scutigeromorphs, the stiletto-like forcipules are only capable of stabbing motion and are solely involved in prey envenomation. In the remaining four orders, the forcipules bear a cutting edge on the inner curvature of the tarsungulum which allow for the dissection and the mastication of prey items.

Scutigeromorphs are fast-running ambush predators using their long legs to "cage" their prey before stabbing it with a vertical motion of the forcipules. Prey are located

through olfactory and tactile cues and attacks usually take place on open grounds (Voigtländer 2011). The diet of the common house centipede *Scutigera coleoptrata* is composed of a variety of flying and terrestrial arthropods, including spiders and small centipedes (Lewis 1981). The species demonstrates territorial habits, with spatial segregation between males and females (Lewis 1981). Cannibalism is frequent, and adult females have a particular liking for freshly molted males (Lewis 1981).

Lithobiomorphs are opportunistic ambush and foraging predators, locating their prey through direct contact with their legs or their antennae (Voigtländer 2011). Prey are seized with the forcipules and kept firmly close to the mouth. Although most lithobiomorphs hunt small prey under the cover of the leaf litter, stones, and logs, some species are known to climb trees in search of small insects (Voigtländer 2011). In British woodlands, a large array of insects, arachnids, and worms seem to constitute the diet of *Lithobius forficatus* (Lewis 1981).

Scolopendromorphs prey mainly upon arthropods, but large specimens are known to occasionally feed on small vertebrates (bats, Molinari et al. 2005; toads, Carpenter and Gillingham 1984; rodents, Clark 1979; snakes, Okeden 1903). The Chinese red-head centipede *Scolopendra subspinipes mutilans* is capable of choosing prey depending on the amount of venom available in its venom glands and the sensitivity of the prey to its venom, thus suggesting the presence of a complex prey detection system. The prey is usually manipulated and oriented before envenomation which usually occurs in the head or thorax (Fig. 6) (Dugon and Arthur 2012b). Although a few species of scolopendromorphs have shown aggregation behavior in captivity (e.g., *Scolopendra subspinipes mutilans*, *Alipes grandidieri*), most scolopendromorphs are solitary and highly territorial. Intraspecific and intergenerational cannibalism is common (Siriwut et al. 2014) and may be involved in the regulation of population density.

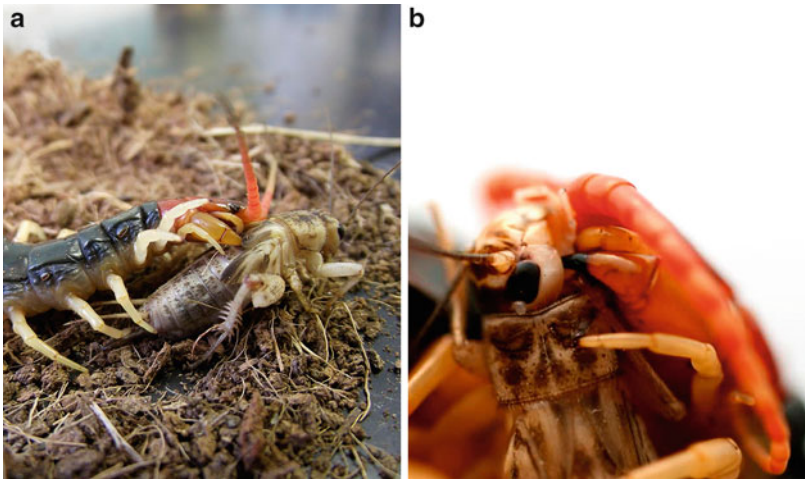

Fig. 6 *Scolopendra subspinipes mutilans* injecting venom in (**a**) the thorax and (**b**) the head of field crickets *Gryllus assimilis*

Geophilomorphs are opportunistic burrowing predators of the topsoil layers. These blind centipedes rely on the mechanoreceptors and chemoreceptors located on their antennae and forcipules to locate their prey at a distance. Geophilomorphs have been observed feeding on small arthropods, including ants, woodlice, coleopteran larvae, and dipteran larvae, but small earthworms are likely to make the bulk of their diet (Lewis 1981). Reports suggest that they occasionally feed on plant material (Voigtländer 2011), although this intake may be marginal. *Strigamia maritima*, a North-Atlantic littoral species of the supra-tidal fringes, forms colonies of hundreds of specimens and has been observed feeding in groups of up to 20 individuals (Lewis 1981). Geophilomorphs use their strong forcipules and cephalic shield to cut through the cuticle of prey and insert their head and anterior trunk segment to devour the prey from the inside (Lewis 1960).

Conclusion and Future Directions

The forcipular apparatus of centipedes is a synapomorphic character which was already possessed by the genus *Crussolum* during the Late Silurian (418 MY). It most likely predates the split between Notostigmophora and Pleurostigmophora which may have occurred 430–450 million years ago (Murienne et al. 2010). If the venom gland was already active at this point – and it may have been – centipedes could compete for the title of "oldest terrestrial venomous animal" alongside archaic arachnids (Undheim et al. 2014).

Evidence tends to demonstrate that the forcipular apparatus is the result of the individualization of the first trunk segment. Because of its strategic post-cephalic position, this segment may have been under an important selective pressure. It was first reassigned to perform a multitude of novel tasks, from preening to prey capture and feeding. Once established in these roles, it probably underwent further specialization when centipedes diversified and occupied new ecological niches, from open spaces to subterranean crevices.

The venom glands may have evolved shortly after the reassignment of the forcipules from locomotory to prey-seizing appendages. The venom glands start developing on the dorsolateral part of the forcipules' apical claws and then penetrate the forcipules more posteriorly, down to the trochantero-prefemurs.

The broad developmental sequence of the forcipules can be summarized in three major steps: (1) the forcipular segment develops following the general anteroposterior segmental direction; (2) the forcipules gain the typical antero-median orientation at a late developmental stage; and (3) the venom gland develops once the forcipules are already formed into sclerotized prehensile appendages and before the centipede starts to hunt.

The presence of nerve endings in the core of the venom gland suggests that (1) the animal can possibly regulate the amount of venom it delivers and (2) the animal may "know" how much venom is available in the venom glands and adapt its foraging behavior accordingly. Also, venom is preferably injected into the body parts where it is most efficient (Dugon and Arthur 2012b), thus confirming a parsimonious use of venom.

The current literature on the venom system of centipedes is largely based on the interpretation of morphological observations, ecological studies, and subsequent deductions on the possible evolutionary and developmental trajectories of the apparatus. This approach has its shortcomings, notably the lack of comparative molecular data which would permit insights into the causality of developmental patterns, both general ones and ones that are distinct to each species (e.g., due to heterochrony).

For that reason, further comparative studies of the gene cascade involved in the formation and identity of the forcipular segment are needed. Some of these genes have already been identified for the lithobiomorph *Lithobius atkinsoni* (Hughes and Kaufman 2002) and the geophilomorph *Strigamia maritima* (Brena et al. 2006; Hayden and Arthur 2013). However, information is fragmentary and no in-depth comparative analysis has been performed so far. An insight from the developmental genetics of a scutigeromorph, a craterostigmomorph, and a scolopendromorph would be very valuable.

A second topic of research directly related to the venom apparatus would be an in-depth comparative study of the venom following two approaches: (1) the creation of cDNA libraries to investigate the venom gland transcriptomes across the five orders and trace back the evolution of the venom gland on the basis of molecular evidence and (2) an assessment of the spectrum of venom components, not only between medically significant species but also at the inter-order, intrageneric, and intraspecific levels. Such population venomic studies may shed some light on the interactions between occupation of ecological niches, venom evolution, and speciation events.

While functional venom studies trigger the interest of both the academic world and the pharmaceutical industry, the evolution of venom systems in invertebrate organisms has attracted relatively little attention so far. Here, there is a virtually untapped potential for important discoveries in many animal phyla. Such work would produce very interesting comparative material to address conceptual questions related to evolutionary novelties, gene co-option, and the functional shift of preexisting structures. Also, from a more pragmatic perspective, a better understanding of the origin, evolution, and development of venom systems would profit applied research by providing a new insight into the evolution of complex proteins and the way they are produced.

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Evolutionary History of Venom Glands in the Siluriformes

14

Jeremy J. Wright

Abstract

The order Siluriformes represents a hyperdiverse group of fishes (>3,000 currently recognized species), which has been known to contain venomous species diversity for over 250 years. In spite of this historical knowledge, scientific examinations of the basic characteristics and evolutionary history of these species' venom glands, and their products, have been extremely sparse compared to those of terrestrial venomous organisms, or even venomous fishes in general. Here, the current state of knowledge regarding the venom glands of catfishes and their products is examined in a review of morphological, pharmacological, and chemical studies of these structures. Several hypotheses regarding the evolution of siluriform venom glands are able to be drawn from the information contained in these studies as well as the limited work that has attempted to study the evolution of these structures in detail. These include selective scenarios to explain the secondary losses of venom glands in several catfish species and families, compositional variation in siluriform venom chemistry, and the derivation of venom glands from secretory cells of the epidermis. Future work directly addressing multiple issues of venom production and composition in catfishes is necessary before investigations of the evolution of siluriform venoms and delivery structures can reach the levels of detail and sophistication seen in other venomous groups. These studies will benefit greatly from the advent of genomic, transcriptomic, and proteomic methods, which have seen wide use in examinations of venoms produced by other taxa, but have yet to be widely applied to analyses of piscine venoms.

Keywords

Catfish • Defense • Proteins • Epidermal • Crinotoxins

J.J. Wright (✉)
New York State Museum, Albany, NY, USA
e-mail: Jeremy.Wright@nysed.gov

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_9

Contents

Introduction

Species falling under the general classification of "fishes" (a paraphyletic assemblage including the classes Myxini (hagfishes), Petromyzontida (lampreys), Chondrichthyes (sharks, rays, and chimaeras), Actinopterygii (ray-finned fishes), Sarcopterygii (coelacanths and lungfishes)) represent more than half of the world's known vertebrate species (Nelson 2006). Many species within the Chondrichthyes and Actinopterygii have long been known to utilize venoms in a natural defensive capacity as well as in interactions with bathers and fishermen (Halstead 1988). Human envenomations by fishes are a relatively common occurrence; globally, incidents involving venomous spiny-rayed fish species (superorder Acanthomorpha) alone number over 50,000 cases annually (Smith and Wheeler 2006), which, due both to unreported incidents and exclusion of several venomous groups, likely severely underestimates the actual number of cases. In one study, nearly 70% of marine fish and 90% of freshwater fish envenomations of humans were caused by non-acanthomorph species (Haddad and Martins 2006); when extrapolated to global estimates, this would elevate the estimated number of incidents to over 100,000 per year.

As would be expected of a venom whose putative purpose is the rapid deterrence of predators, the most common result of envenomation by fish species is intense pain that is highly disproportionate to the magnitude of the injury, suggesting that components of these venoms target nociceptive sensory neurons (Church and Hodgson 2002; Trim and Trim 2013). In addition to the elicitation of this intense pain response, fish venoms are known to cause a number of other physiological symptoms, including cardiovascular, hemolytic, and neuromuscular effects (Halstead 1988; Church and Hodgson 2002; Sivan 2009). Despite their clear

ramifications for human health, fewer than a dozen toxic compounds have been characterized from this highly diverse assemblage (Halstead 1988; Church and Hodgson 2002; Smith and Wheeler 2006). In addition to medical interest in their native physiological effects, fish venoms represent an untapped reservoir of potentially pharmaceutically valuable compounds, particularly as lead compounds in the development of new analgesics, due to their possible ability to directly interact with neuronal signaling pathways (Trim and Trim 2013).

Until recently, however, even the most basic information regarding venomous fishes, such as the number and phylogenetic distribution of venomous taxa, has been unavailable to researchers interested in the evolutionary history of these compounds and the structures that produce them. In the last decade, phylogenetic analyses of acanthomorph species have estimated that 585–650 of the species in this group should be presumed to be venomous, a substantial increase from previous estimates of approximately 200 species (Halstead 1988; Smith and Wheeler 2006). When other types of venomous fishes (Chondrichthyes, Siluriformes) are included, this estimate potentially increases to over 2,500 species, or just under 10% of all known fish species (Wright 2009). Though this level of species diversity is greater than that of all other venomous vertebrates combined, venomous fishes remain severely understudied relative to venomous terrestrial organisms, as evidenced by a recent review of venom evolution that mentions fishes only in passing, and without providing any detailed information regarding the toxic action of their venoms (Casewell et al. 2012).

The order Siluriformes, commonly known as catfishes, is a globally distributed, highly diverse clade containing over 3,000 currently recognized species in 36–38 families (Sullivan et al. 2006; Ferraris 2007). The order has been known to contain venomous representatives for nearly 300 years, beginning with Johann Richter's description of Spanish fishermen's fear of stings from marine catfishes belonging to the family Ariidae (Halstead 1988). Although the stings of most catfish species are relatively harmless, albeit very uncomfortable, fatalities have been reported as the result of envenomations by members of the families Plotosidae (*Plotosus lineatus*) and Clariidae (*Heteropneustes fossilis*) (Halstead 1988). These species undoubtedly possess notably potent venoms, but these fatalities, which occurred in the late nineteenth and early twentieth centuries, were likely due to poor medical care and/or secondary infection of the wound (a common complication of siluriform envenomations) (Halstead et al. 1953; Haddad and Martins 2006). Only one modern fatality involving a catfish sting has been recorded, a freak accident in which a fisherman's heart was penetrated by the spine of a large individual (Haddad et al. 2008).

While venomous fishes in general have received little research attention relative to other venomous groups of organisms, catfishes in particular have suffered from a dearth of focused studies. Until recently, few families had been confirmed to contain venomous species, although several had been suspected to harbor venom-producing representatives (Halstead 1988). Wright (2009) performed an extensive histological survey of nearly 150 catfish species, sampling over 100 genera (~25% of the genus-level diversity in the order) in 32 families, demonstrating the presence of venomous

Table 1 Taxonomic distributions and estimates of venomous catfish diversity. Estimates reproduced from Wright (2009)

Taxon	# Presumed venomous
Siluriformes – catfishes	≈1,250–1,625 species
Akysidae – Asian stream catfishes	48
Amblycipitidae – torrent catfishes	26–28
Anchariidae – Madagascan catfishes	4–6
Ariidae – sea catfishes	67–134
Bagridae – bagrid catfishes	176–198
Callichthyidae – armored catfishes	182–194
Chacidae – angler catfishes	3
Clariidae – labyrinth catfishes	79–114
Claroteidae – claroteid catfishes	56–84
Cranoglanididae – armorhead catfishes	3
Doradidae – thorny catfishes	48–81
Heptapteridae – shrimp catfishes	91–160
Ictaluridae – North American catfishes	57–64
Mochokidae – squeakers	166–189
Pangasiidae – shark catfishes	27–30
Pimelodidae – antennae catfishes	41–79
Plotosidae – eel-tailed catfishes	17–37
Pseudopimelodidae – bumblebee catfishes	21–31
Schilbeidae – glass catfishes	48–62
Siluridae – sheat catfishes	74–83

taxa in 20 siluriform families (Table 1), and arriving at a total estimate of 1,250–1,625 venomous species, a significant majority of venomous actinopterygian diversity. The upper end of this estimate would make catfishes the most diverse single group of venomous vertebrates known (Wright 2009; Egge and Simons 2011) and continues to increase each year, due to descriptions of new species in venomous families and genera.

Examinations of the evolutionary history of venoms and venom production in catfishes are currently hampered by a lack of resolution in higher-level siluriform phylogeny (Sullivan et al. 2006) and basic knowledge regarding the identity of venom components and the genetic architecture underlying their production (Wright 2009; Egge and Simons 2011) as well as selective factors driving the compositional evolution and properties of defensive venoms (Casewell et al. 2012). Nonetheless, sufficient progress has been made to be able to generate inferences regarding several aspects of catfish venom gland evolution. This chapter attempts to provide a review of our current knowledge and hypotheses regarding the evolution of siluriform venom glands, as developed through an examination of relevant literature concerning the identification and anatomy of siluriform venom glands and delivery systems; the toxicology, pharmacology, and basic chemistry of the venoms of species investigated thus far; and the few studies that have attempted to directly address the ecology and evolution of the venom systems of catfishes. Such a survey

serves as an illustration of not only the surprising amount of evolutionary information that can be gleaned from the existing literature but how far the study of venomous catfishes, and venomous fishes in general, must proceed before reaching the levels of detail and sophistication seen in other groups of venomous organisms.

Siluriform Venom Gland and Delivery System Morphology

Gross Morphology

Venoms, by definition, require a method by which their bearer is able to introduce them into the body of a target organism. In all known venomous fishes (with the exception of *Meiacanthus* sp. and members of the deep-sea family Monogathidae), this is accomplished via spiny elements associated with the fins and/or opercular and cleithral bones (Halstead 1988; Smith and Wheeler 2006). These spiny elements contain grooves that facilitate the flow of venom along the spin; in most cases, the glandular tissue rests within the groove itself. The association of these venom glands with spiny elements led Perrière and Goudey-Perrière (2003) to name their toxic secretions acanthotoxins. In *Meiacanthus* sp. (saber-toothed blennies), injection is achieved by the use of enlarged fangs in the bottom jaw rather than spines, with buccal venom glands surrounding the proximal two thirds of the fang (Halstead 1988; Smith and Wheeler 2006). Venom flows toward the site of envenomation through grooves along the anterior fang margins. Monognathids, which lack upper jaws, apparently inject venom via a single, hollow rostral fang, which has paired glands at its base (Bertelsen and Nielsen 1987). These species are unique among venomous fishes, in that they appear to use their venoms to subdue their prey, shrimps that are very large relative to their own size (Bertelsen and Nielsen 1987), and which would have the potential to cause significant damage to these relatively fragile fishes.

The venom glands of catfishes are composed of aggregations of glandular cells associated with bony spines in the dorsal and pectoral fins (Fig. 1a–c), which can be erected and locked into place via frictional forces and/or muscular action when the fish is threatened, effectively increasing the individual's cross-sectional area and leading to increased handling difficulty for potential predators (Bosher et al. 2006; Fine et al. 2011; Emmett and Cochran 2010; Wright 2012a). The pectoral and, occasionally, dorsal spines of many species are additionally armed with retrorse serrations along one or both of the spine margins (Fig. 1b), the presence and orientation of which can vary both between and within different catfish families (Wright 2009; Egge and Simons 2011). When the spine enters a potential predator, the glands are torn, releasing the largely proteinaceous venom into the wound.

This passive method of venom delivery appears to represent a rather primitive condition, which is found across multiple groups of venomous fishes; members of only a few families (e.g., Batrachoididae, Scorpaenidae) show a more specialized, hypodermic-style apparatus characteristic of most other venomous vertebrates (Birkhead 1972; Halstead 1988; Smith and Wheeler 2006). It also results in potentially significant damage to the integumentary and glandular tissue surrounding the

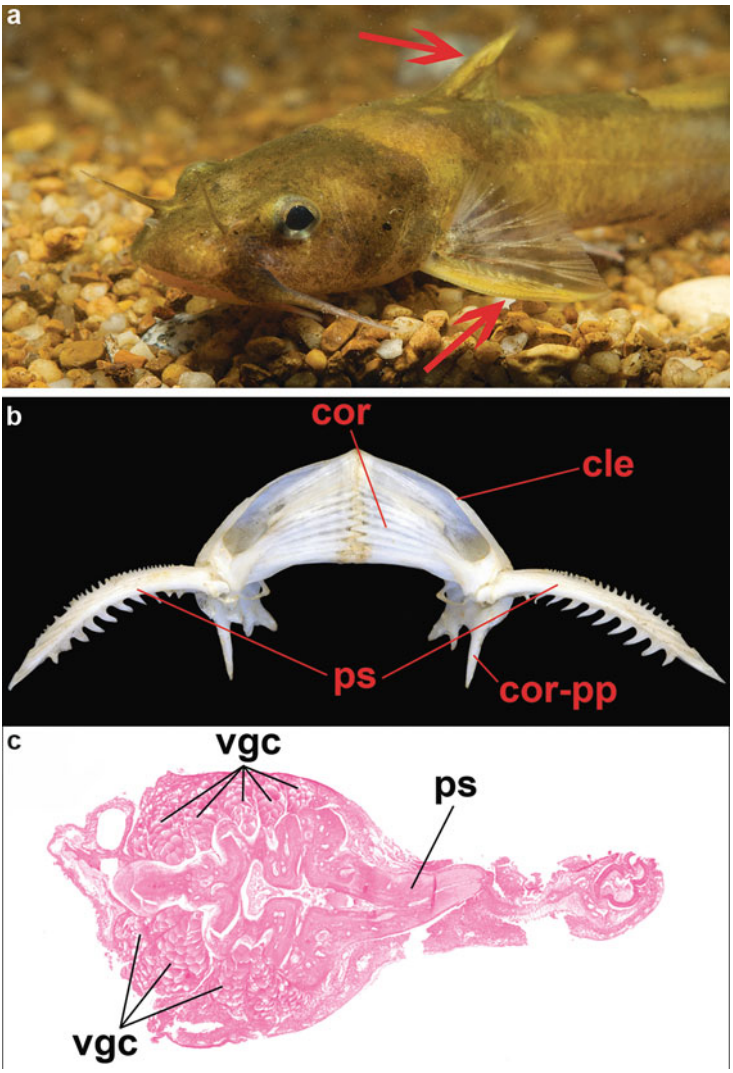

Fig. 1 The venom delivery system of catfishes. (**a**) The venomous species *Noturus stigmosus* (Northern madtom), with *red arrows* indicating the position of the dorsal and pectoral fin spines. (**b**) The pectoral girdle of *N. stigmosus* with articulated fin spines, illustrating the increased levels of spine serration found in this species. (**c**) Cross section of the pectoral fin spine of *N. stigmosus*, showing the association of venom glands with the fin spine. Abbreviations: *ps* pectoral fin spine, *cle* cleithrum, *cor* coracoid, *cor-pp* posterior process of coracoid, *vgc* venom gland cells (Figure reproduced from Wright (2009))

spine, which can take a significant amount of time (over a week) to heal (Birkhead 1972). Nonetheless, such compromised spines still represent a potent antipredatory defense; multiple experiments presenting North American catfish species (family Ictaluridae) to largemouth bass (*Micropterus salmoides*), a common piscivorous

species, have demonstrated that the presence of spines increases predator handling time and catfish survivorship relative to individuals in which the spines had been removed (Bosher et al. 2006; Emmett and Cochran 2010; Wright 2012a). Wright (2012a) further demonstrated, however, that the presence of venom glands significantly increases the antipredatory capabilities of spines in intact tadpole madtoms (*Noturus gyrinus*), relative to individuals in which the venom glands had been surgically removed.

The extent and orientation of the venom glands in relation to spine serrations, as well as grooves within the spine itself, varies significantly between different catfish species (Halstead 1988; Wright 2009; Egge and Simons 2011). Serrated spines may increase the amount of mechanical damage produced when the spine enters a potential predator, increasing the surface area exposed to the concomitantly released venom (Reed 1907; Birkhead 1972; Egge and Simons 2011). There is little evidence, however, to suggest that the venoms of species with greater levels of spine ornamentation possess significantly greater toxicity (Birkhead 1972), nor have experiments been performed to demonstrate increased predator deterrent ability in those species. In fact, Egge and Simons (2011) found that of five evolutionary changes in sting morphology in the genus *Noturus*, four involved decreases in morphological complexity, including the loss of spine serrations, loss of venom gland tissue associated with serrations, or, in one case, the total loss of the venom gland.

These results suggest that certain ecological and life history traits may result in the relaxation of selective pressures related to the maintenance of venom glands, leading to their eventual loss, which appears to be a relatively widespread phenomenon throughout the order (Wright 2009). Such scenarios may include ontogenetic loss of venom glands in species obtaining body sizes that effectively protect them from natural, gape-limited predators (Egge and Simons 2011) or the secondary loss of venom glands in members of families that have lost ossified fin spines (e.g., Malapteruridae, many amphiliids) and, thus, an effective delivery system for metabolically expensive venom compounds (Wright 2009). Wright (2009) also found that members of the families Sisoridae and Erethistidae have secondarily lost venom glands, while maintaining their fin spines. Many of the species in these families occupy highly rheophilic habitats (as does *Ameiurus brunneus*, another species that has secondarily lost venom glands), where effective foraging by large-bodied predatory species would be highly difficult, if not impossible, offering a possible explanation for the lack of venom production in these species.

Cellular Morphology

The cellular morphology of venom glands in fishes is very similar across broad taxonomic categories, indicating possible widespread convergent evolution of these cells. Venom-producing cells are enclosed within an integumentary sheath composed of epithelial cells. The venom gland cells are large and polygonal, with prominent nucleoli and highly granulous cytoplasm, presumably due to high concentrations of venomous peptides (Reed 1907; Halstead et al. 1953; Halstead 1988); in catfishes, the cells of the venom gland are also binucleate (Reed 1907; Halstead

et al. 1953; Halstead 1988). As the cells mature, organelles and nuclear structures are lost and only the cytoplasmic granules are visible. Venomous secretions are either held within the cells or the cells undergo holocrine secretion, whereby the secreting cells are lysed and release the venomous secretions (along with cellular fragments) into the intercellular space, where they are held until being used.

Cameron and Endean (1973) hypothesized that the venom gland cells of fishes and the acanthotoxins that they contain are evolutionarily derived from the clavate or club cells of the epidermis, which secrete proteins known as crinotoxins (Halstead 1988). While crinotoxic secretions are released into the water when the cells are ruptured, ostensibly to repel predators or fouling organisms (Cameron and Endean 1973), the direct injection of these compounds into other organisms has also been shown to have toxic effects (Al-Hassan et al. 1987; Shiomi et al. 1987, 1988). A preliminary study of the catfish *Plotosus lineatus* offers some support for Cameron and Endean's hypothesis, as the club cells of this species were found to produce a substance that is similar, and possibly identical, to one of the toxic fractions found in the venom gland, based on immunological reactions (Shiomi et al. 1988).

Perrière and Goudey-Perrière (2003), however, point out that common production of a single toxic component is not sufficient evidence to prove the homology of these cell types. While certain crinotoxins and acanthotoxins produced by *P. lineatus* show similar histochemical and pharmacological activities, Whitear et al. (1991a) found distinct differences in the ultrastructure and histochemistry of the venom gland cells and club cells in the skin of *Heteropneustes fossilis* (Indian stinging catfish) that, in their estimation, precludes the homology of the two cell types. Specifically, club cells were found to contain helical filaments and a division of the cytoplasm into perinuclear and peripheral zones, both of which were lacking in the venom cells. Additionally, while a previous study (Zaccone et al. 1990) had shown a positive immunohistochemical reaction for serotonin in the club cells of this species, Whitear et al. (1991a) found that this reaction was lacking in the venom cells.

Whitear et al. (1991a) did not address why these differences should mean that the venom gland cells could not possibly have been derived from epidermal club cells. If venom glands are indeed adaptive structures, one might expect their cellular morphology and the secretions that they produce to be subject to selection pressures that differ from those experienced by secretory cells in other locations. The differences reported by Whitear et al. may simply reflect this history. Additional comparative morphological and transcriptomic studies of venom glands and secretory epidermal cells from different groups of venomous catfishes should serve to clarify these issues.

Siluriform Axillary Glands

Gross Morphology

In addition to the venom glands lining the spinous elements of the fins, many siluriform species possess secretory glands situated in the axil of the pectoral fin (Reed 1907; Halstead et al. 1953; Halstead and Smith 1954; Greven et al. 2006).

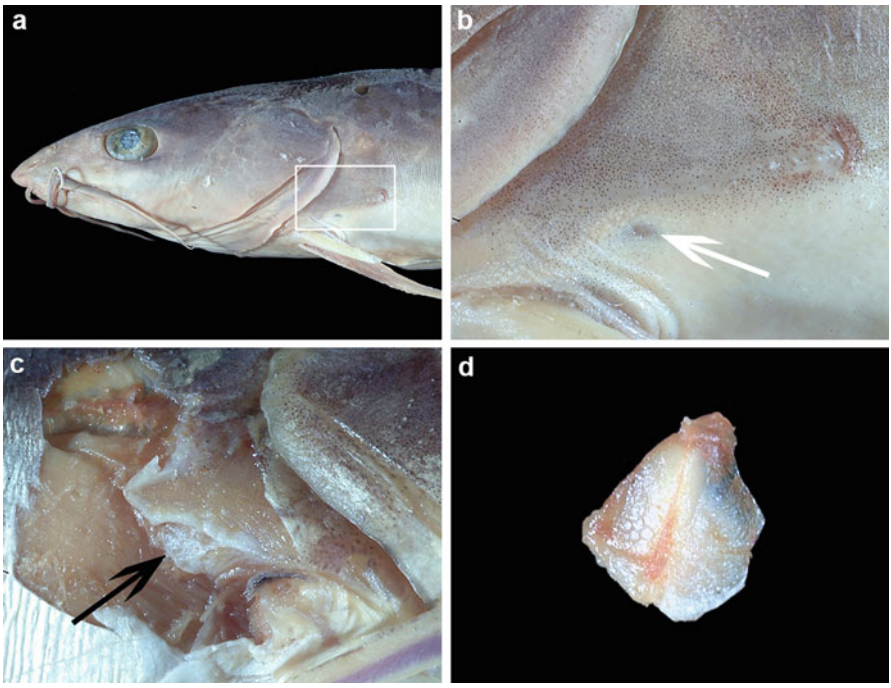

Fig. 2 Gross morphology of the axillary glands and associated structures in catfishes. (**a**) Anterior half of *Ariopsis felis*, with cleithral region and axillary pore indicated by *white box*. (**b**) Close-up of cleithral region from the same specimen, with the axillary pore indicated by the *white arrow*. (**c**) Cleithral region of *Bagre marinus* with skin removed, showing the position of the axillary gland relative to the cleithrum. *Black arrow* indicates glandular tissue, which extends further upward behind the cleithrum. (**d**) The axillary gland of the same specimen, removed from behind the cleithrum

These structures are known from several families, including the Akysidae, Ariidae, Callichthyidae, Ictaluridae, Mochokidae, and Plotosidae, but to date, no comprehensive survey has been performed to document the distribution of axillary glands throughout the Siluriformes. Various authors have considered the axillary glands to be part of the venom apparatus (Reed 1907; Halstead et al. 1953; Birkhead 1967; Cameron and Endean 1971), and, as such, they are briefly discussed here.

The axillary glands of catfishes are small pouch-like structures that release their secretions via a pore located below the postcleithral process, near the base of the pectoral fin spine (Fig. 2a, b). In most species, the gland itself is roughly triangular in shape, with its upper half covered by, and the long axis oriented at a perpendicular to, the postcleithrum (Fig. 2c, d). The interior of the gland is divided into several lobes, with each lobe being separated from the others by a layer of connective tissue (Reed 1907; Halstead et al. 1953). Recent studies of callichthyid catfishes have revealed a simple, tubular morphology of the axillary gland in these species (Greven et al. 2006).

Cellular Morphology

The secretory cells of siluriform axillary glands are located within further subdivisions of the axillary gland lobes (Fig. 3a). In all species thus far studied, these cells are large and polygonal and contain large quantities of a granular, secretory product, which has been shown by multiple authors to be proteinaceous in nature (Cameron and Endean 1971; Al-Hassan et al. 1987; Kiehl et al. 2006). The cellular ultrastructure resembles that of the venom gland, with the cells originating as binucleate cells with prominent nucleoli and large amounts of endoplasmic reticulum (Whitear et al. 1991b). The cells become completely filled with secretory product as they mature, to the point that most subcellular structures are no longer visible (Halstead et al. 1953; Cameron and Endean 1971; Kiehl et al. 2006) (Fig. 3b). Release of the secretory product appears to be holocrine in nature, which is indicated by the presence of burst cells in secretions drawn directly from the axillary pore (Reed 1907; Cameron and Endean 1971; Whitear et al. 1991b) and lack of evidence for other methods of secretion.

Possible Function

The earliest mention of axillary glands in catfishes was made by Günther (1880). He assumed that secretions issuing from the axillary pore anoint the pectoral fin spine, allowing them to be injected along with secretions from the pectoral venom glands.

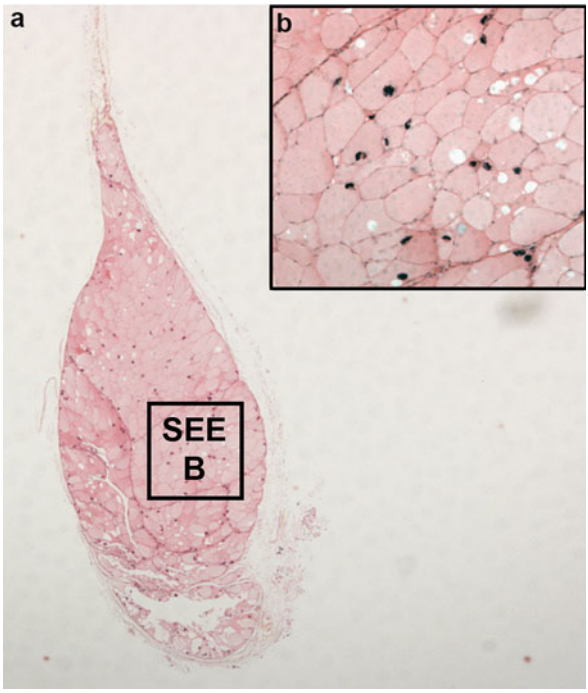

Fig. 3 Cellular morphology of the axillary gland of *Bagre marinus*. Photomicrographs of (**a**) a histological section of the axillary gland pictured in Fig. 2d and (**b**) a close-up view of the glandular cells

Many works that followed (i.e., Reed 1907) accepted this statement without experimental confirmation. More recently however, several additional, more likely, hypotheses have been proposed for the function of these structures. While later studies showed that axillary gland extracts are toxic when injected into other organisms (Cameron and Endean 1971; Birkhead 1967), the water-soluble nature of axillary pore secretions is difficult to reconcile with the venomous scenario envisioned by earlier authors. Current hypotheses regarding the function of the axillary gland secretions include antimicrobial (Kiehl et al. 2006), ichthyotoxic (Greven et al. 2006), pheremonal, and ionoregulatory roles, though only the first two are supported by empirical evidence.

While it appears that the axillary glands of catfishes do not function as part of the venom delivery apparatus, their true function and the action of their products remains a potentially fruitful area for future research. Fairly simple procedures, such as comparative electrophoresis, HPLC, or mass spectrometry of venom and axillary gland extracts, could be used to more conclusively rule out the presence of axillary gland secretions on the pectoral spine. Further investigations of the antimicrobial and ichthyotoxic hypotheses that have thus far received preliminary support are also warranted.

Pharmacology and Toxicology of Siluriform Venoms

Wright (2009, 2011, 2012a, b) has demonstrated that the crude venom extracts of a phylogenetically diverse group of catfish species produce a wide array of symptoms when injected into a model predatory species (largemouth bass), most notably the rapid loss of color pattern throughout the body, which has been observed from the venoms of nearly every catfish species studied thus far. This suggests the presence of conserved venom function that acts in some way on the nervous system, which controls chromatophore and melanophore activity. Suites of additional envenomation symptoms observed by Wright (2009, 2011, 2012a, b) were highly species specific and included the expansion of melanophores at the injection site, rapid loss of color pattern elsewhere on the body, muscle spasms of varying degrees of intensity and duration, hemorrhage, loss of equilibrium, and, in the case of *Plotosus lineatus*, rapid mortality. Earlier work by Birkhead (1967, 1972) examined the venoms of several species from the North American family Ictaluridae that were also studied by Wright (2012b) and found that they produced some of the same symptoms, including melanophore expansion and hemorrhage. Several additional symptoms were found, however, including notable edema, necrosis, and death. Some of these differences may be attributable to Birkhead's use of a different assay organism (*Gambusia affinis*) in his assessments of venom toxicity. It must be noted, however, that the effects demonstrated by both Birkhead (1967, 1972) and Wright (2009, 2011, 2012a, b) were likely elicited by the injection of much higher doses of venom than would be encountered in a natural situation. These encounters usually result in violent ejection of the catfish from the buccal cavity of the bass, accompanied by rapid gaping of the mouth and flaring of the gills (Wright 2011, 2012a).

As naturally occurring substances which are able to elicit potent responses in vertebrate physiological systems, the venoms of fishes have come under increased scrutiny as possible sources of future biomedical compounds. Studies of the toxic effects elicited by fish venoms in other organisms have revealed a high degree of similarity in these effects and the mechanism of their production, providing an additional example of apparent convergent evolution of fish venom glands and the substances they produce. The most common sites of human envenomation are the hands or feet, and in many cases, the pain has been known to travel up the entire length of the affected appendage (Halstead et al. 1953; Calton and Burnett 1975; Halstead 1988; Church and Hodgson 2002; Sivan 2009). The pharmacological actions of the venoms of a select few catfish species have been studied and have been shown to have cardiovascular, neuromuscular, and general cytolytic effects in various assays (Church and Hodgson 2002; Sivan 2009).

The widespread elicitation of cardiovascular effects by piscine venoms in experimental tissue preparations indicates convergence in venom target systems, although the nature of these effects and the mechanisms by which they are produced vary between species and taxonomic groups. The venoms of *Plotosus canius* and *Heteropneustes fossilis* are thought to either contain or cause the release of prostaglandins, contributing to their production of smooth muscle contractile responses in a number of tissue preparations (Church and Hodgson 2002; Sivan 2009). In contrast, the smooth muscle contraction produced by the venom of *Arius thallasinus* appears to be produced through effects on muscarinic acetylcholine receptors (Church and Hodgson 2002; Sivan 2009). Effects on cardiac muscle preparations are similarly variable, with the venom of *H. fossilis* producing inotropic increases in guinea pig and toad hearts, while toxin-PC isolated from *P. canius* causes cessation of heartbeat in guinea pig preparations (Auudy and Gomes 1996; Church and Hodgson 2002; Sivan 2009). The combined effects of siluriform venoms on blood vessel and cardiac function have also produced alternate results in in vivo preparations. The venom of *P. canius* has been shown to produce a hypertensive response, while that of *H. fossilis* produces a hypotensive effect (Auddy and Gomes 1996; Church and Hodgson 2002; Sivan 2009).

Potent neuromuscular activities have been reported from several catfish venoms, in addition to the systemic, neurologically mediated color loss and muscle spasms observed in toxicity assays using living predators. The crude venom of *Plotosus canius* has been shown to irreversibly inhibit electrically induced muscle contractions in rat and chick muscle preparations, as has an isolated preparation of toxin-PC, the lethal component of that species' venom (Church and Hodgson 2002; Sivan 2009). It is thought that toxin-PC prevents neurotransmitter release presynaptically, as it produces sustained muscular contraction without affecting muscular preparations' responses to acetylcholine or carbachol, although its blockage of neuromuscular activity apparently does not result from K^+-channel or cholinesterase modulating abilities (Auddy and Gomes 1996; Church and Hodgson 2002; Sivan 2009). The venom of a related species, *P. lineatus*, has also been shown to produce neurotoxic symptoms upon intraperitoneal injection into mice (Fahim et al. 1996). In another case of interspecific divergence in siluriform venom effects, however the

venom of *Heteropneustes fossilis* has been found to display no appreciable neuromuscular effect (Church and Hodgson 2002; Sivan 2009).

Nearly all piscine venoms exhibit cytolytic properties, and the venoms of catfishes are no exception. In fact, local necrosis is one of the most common clinical symptoms of piscine envenomations (Sivan 2009) and has also been documented in Birkhead's (1967, 1972) envenomations of *Gambusia* with ictalurid species' venoms. The lack of such symptoms in Wright's (2009, 2011, 2012a, b) experiments, however, may indicate that these necroses are largely due to secondary bacterial infections. Nonetheless, the venoms of several siluriform species have produced hemolysis in rabbit (*Plotosus canius*), rat (*P. canius*, *P. lineatus*), human (*Arius thallasinus*), mouse (*P. canius*), cow (*A. thallasinus*, *P. canius*), and sheep (*A. thallasinus*) erythrocytes (Church and Hodgson 2002). The venom of *P. lineatus* has additionally been shown to be cytotoxic to cultured Ehrlich ascites tumor cells as well as a number of other cell types (Fahim et al. 1996). The cytolytic action of these venoms is thought to contribute to other negative effects of envenomation, through forming pores in the plasma membranes of target cells, allowing the influx of Ca^{2+} which triggers the release of several biologically active compounds from the cell (Church and Hodgson 2002). Such an action is also known from bee (Pawlak et al. 1991) and platypus venoms (Kourie 1999), both of which are primarily pain-producing venoms, like those of catfishes.

Chemistry of Siluriform Venoms

Proteins

The majority of existing information regarding the toxic proteins found in siluriform and other piscine venoms concerns the sizes of these compounds in various species. Of the ten fish species' venoms detailed by Church and Hodgson (2002), the sizes of the toxic compounds ranged from 15 to 324 kDa. Catfish venoms generally fall within the lower end of this range (10–15 kDa) (Calton and Burnett 1975; Auddy and Gomes 1996), although Wright (2009) identified an additional putative toxin of approximately 110 kDa in the venoms of several species. Siluriform venoms appear to display a high degree of conservatism in at least some of their toxic components, as this putative 110 kDa toxin has been found in the venom electrophoretic profile of nearly every siluriform species thus far examined (Wright 2009, 2011, 2012a, b; Fig. 4). Without additional information regarding the actual amino acid sequence and structure of this protein, however, it is not possible to state conclusively that the identity of this venom protein is the same between all species in which it has been found. Nonetheless, the widespread presence and apparent conservation of a toxic peptide of this size in catfish venoms indicates that these proteins are likely to be involved in the rapid loss of coloration seen when a natural predator is injected with catfish venom extracts (Wright 2009, 2011, 2012a, b).

Additional putatively toxic peptides, generally falling within the size range of 10–20 kDa (Calton and Burnett 1975; Church and Hodgson 2002; Auddy and

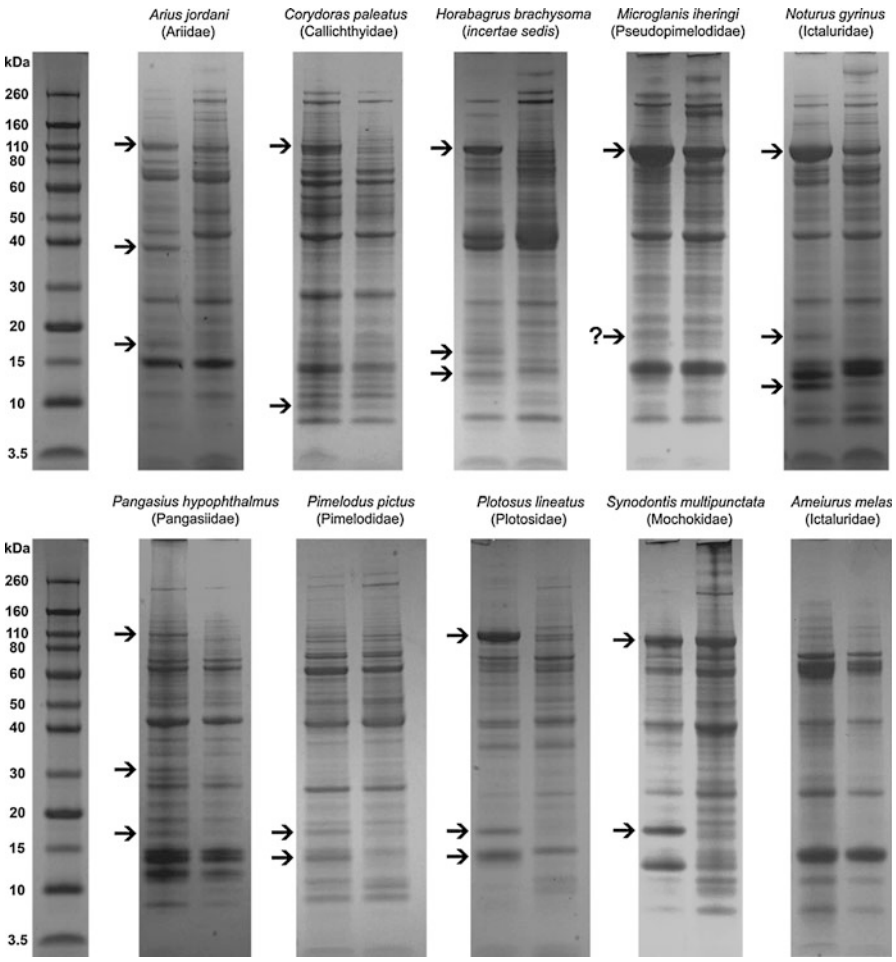

Fig. 4 SDS-PAGE profiles of venom extracts from several catfish species. *Left lanes* represent venom extracts, *right lanes* represent extracts prepared from fin tissue. *Arrows* indicate positions of unique venom protein bands or proteins found in greater concentrations in venom extracts than in fin tissue extracts. (?) represents ambiguity between smearing and an additional, unique venom peptide band. Large quantities of a 110 kDa peptide are found in the venom extracts of nearly all species shown, with the exception of *Pimelodus*. The presence and variation of venom peptides in the size range of 10–20 kDa is also clearly visible. Samples from non-venomous *Ameiurus melas* are shown for comparison (Figure reproduced from Wright (2009))

Gomes 1996; Wright 2009), have been identified in the venoms of many siluriform species, although putative toxins of 40–50 kDa have been indicated in some ariid species (Junqueira et al. 2007; Wright 2009). The lethal fraction of the venom of *Plotosus canius* (toxin-PC) is one such protein, having a molecular weight of approximately 15 kDa (Auddy and Gomes 1996; Wright 2009). These smaller venom components show significant variation in number and size over interspecific,

intergeneric, and interfamilial scales, identifying them as likely candidates underlying the variation observed in the effects elicited by different species' venoms in toxicological assays (Wright 2009, 2012b). This high degree of variation, even between relatively closely related species, would seem to strongly indicate that selective forces associated with different habitats and/or predatory regimes have contributed to the establishment of differing levels of venom protein identity and complexity between different siluriform lineages. Our current lack of information regarding the genes coding for these proteins, as well as their structure and physiological targets, precludes the testing of further hypotheses regarding the evolution of these compounds. The molecular weight data obtained thus far for siluriform venoms is nonetheless valuable, as it offers an independent check on the identities of the potentially novel toxin-related sequences that will undoubtedly be uncovered by future evolutionary studies utilizing omics-scale technologies and analytical methods to identify catfish venom toxin genes, transcripts, and proteins.

Chemical Complexity of Siluriform Venoms

In contrast to the venoms of organisms that utilize these secretions in prey capture, which can potentially contain hundreds of toxic components per species, the venoms of fishes and other organisms that utilize venom in a strictly defensive capacity appear to contain only one or a few toxic components (Church and Hodgson 2002; Wright 2009; Casewell et al. 2012). This has been confirmed for several catfish species using comparative electrophoresis of extracts prepared from fin spines and associated tissues, which showed only one to three unique peptides being expressed in spine extracts relative to control extracts prepared from histologically similar fin tissues (Wright 2009, 2011, 2012a, b). The toxic nature of these peptides was confirmed using toxicity assays performed in largemouth bass (*Micropterus salmoides*), which showed marked, species-specific effects associated with injection of fin spine extracts, but no toxicity associated with the injection of fin tissue extracts. An interesting parallel to this condition of reduced venom toxin diversity is found in the venoms of sea snakes, which have also been shown to contain a highly reduced number of toxic components relative to other venomous snakes (Fry et al. 2003). The similarities become even more striking when one considers that venomous marine snakes represent two evolutionary radiations that have independently arrived at a state of reduced venom complexity (Scanlon and Lee 2004), while compositionally simple venoms have been independently derived in acanthomorph fishes no fewer than 11 times (Smith and Wheeler 2006), and at least twice in catfishes (Wright 2009).

The white catfish (*Ameiurus catus*) may represent an exception to the generalization that piscine venoms exhibit low toxin diversity. The venom of this species was found to contain two to eight fractions that showed lethal activity in mice (Calton and Burnett 1975). The additional finding that *A. catus* venom lost little to no activity following treatment with trypsin and elevated temperature indicates that additional, non-proteinaceous compounds may be present in the venomous secretions of this

species. These results are questionable however, as different methods of analysis yielded proteinaceous fractions of varying weights and biological activities. Wright (2012b) also examined the venom of this species, using the comparative electrophoretic methods discussed above, and found evidence for only two putative toxic peptides in prepared fin spine extracts; these produced a significant toxic effect in injected largemouth bass. While this study could not speak to the possible presence of non-proteinaceous toxins in *A. catus* venom, it clearly supports the lower value from Calton and Burnett's (1975) estimate of the number of lethal fractions in the venom of *A. catus* and is much more consistent with what is known from other species. It is possible, however, that siluriform venoms show intraspecific regional variation and that the conflicting results of these studies resulted from drawing individuals from geographically distant populations (Chesapeake Bay tributaries in the case of Calton and Burnett, an inland North Carolina lake in the case of Wright).

The low number of toxic compounds found in fish venoms would appear to be an asset to studies of their evolution, as the problems of homology inherent in evolutionary studies of species that produce many different toxins should be easily addressed. It is tempting to suggest that the parallel streamlining of these species' venoms is due to selection associated with a common target: piscine physiological systems. Little empirical evidence exists to support this hypothesis however, as few studies of the action of sea snake and piscine venoms on their (presumed) natural targets exist. The few studies of sea snake venoms performed in this context have indicated that likely prey species possess high levels of resistance to sea snake venoms (Heatwole and Powell 1998). This would appear to run counter to a selective streamlining hypothesis, as one might expect these species of sea snakes to possess more complex venoms to overcome prey resistances to particular toxic compounds. Preliminary results from studies on ictalurid catfishes (Wright 2012b) indicate that the venoms of bullheads have little effect on potential predators with which they share a habitat type. These results may indicate that coevolution between predator and prey is occurring in these systems, leading to these somewhat counterintuitive results. Further studies are clearly necessary to examine possible correlations between the low number of toxic compounds in catfish venoms and the selective factors influencing siluriform venom evolution.

Evolutionary Origins of Siluriform Venom Glands

Phylogenetic Distribution

Our lack of knowledge regarding basic characteristics of siluriform venoms and their targets represents a significant obstacle to the study of their evolution, which is compounded by our incomplete understanding of siluriform phylogeny. Current classifications divide modern catfishes into two monophyletic suborders, the Loricarioidei (South American armored, sucker-mouthed, pencil, and parasitic catfishes) and the Siluroidei (all remaining catfish families). The morphologically primitive family Diplomystidae has been alternatively recovered as the sister group

to all catfishes, or the sister group of the Siluroidei, with the Loricariodei sister to all other catfishes. Multiple higher-level phylogenetic analyses of catfishes, using both morphological and molecular data (e.g., Diogo 2004; Hardman 2005; Sullivan et al. 2006), are available, but these studies nearly universally suffer from poorly supported resolution of the early evolutionary relationships between catfish families, particularly within the Siluroidei.

Wright (2009) mapped the presence of venom glands (as determined from histological surveys) onto available siluriform phylogenies (Fig. 5), generating

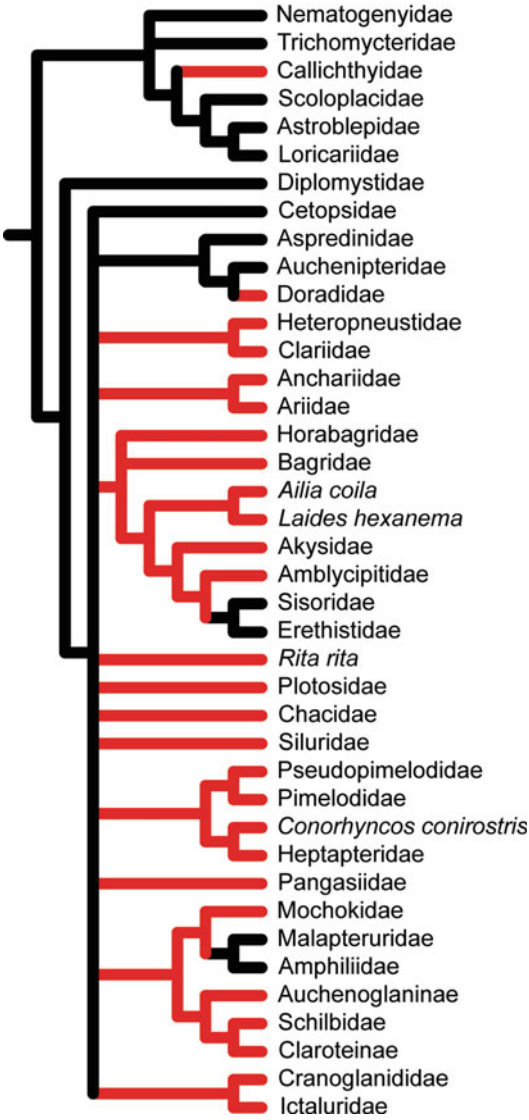

Fig. 5 Results of mapping the presence of venom glands onto a siluriform molecular phylogeny. Phylogeny from Sullivan et al. (2006). *Red* branches indicate venomous lineages; *black* branches indicate non-venomous lineages. An independent origin of venom glands in the Callichthyidae is clearly supported. The possible independent origin of venom glands in the Doradidae is also depicted. The evolutionary history of venom glands at the base of the Siluroidei is obscured, due to poor phylogenetic resolution, but a single origin in the early history of the suborder remains the most parsimonious hypothesis (Figure reproduced from Wright (2009))

reasonable inferences regarding the number of times venom glands have been evolved within the order, in spite of these poorly resolved basal relationships. The presence of venom glands in the Callichthyidae almost certainly represents an independent origin of venom glands within the Loricarioidei, as none of the other families within the suborder showed any evidence of venom gland tissue associated with their fin spines. Venom glands are widespread in the Siluroidei (19 of the 20 known venomous siluriform families), indicating that a single, relatively basal origin of venom glands within this suborder is the most parsimonious hypothesis, although the exact evolutionary placement of this event awaits further resolution of relationships within the clade. Sullivan et al.'s (2006) proposed phylogeny requires a third evolutionary derivation of venom glands in the South American family Doradidae due to the recovery of this family in a sister relationship with the nonvenomous Auchenipteridae, within a clade also containing the Aspredinidae, another nonvenomous family. The venom glands of doradids do vary significantly from those of other siluroid groups in terms of their organizational structure, orientation relative to the fin spine, and visibility without magnification, offering morphological support for a hypothesis of an independent redevelopment of venom glands within the family following their secondary loss during the origins of this clade.

Evolution from Epidermal Secretory Cells

There is strong evidence that the venom glands of several previously studied catfish species produce similar compounds to epidermal glandular cells. Immunocytochemical assays of epidermal cells taken from *Plotosus lineatus* have indicated that these cells produce a highly similar protein to one of the toxic fractions identified from the venom gland of that species (Shiomi et al. 1988). Further evidence for this similarity is provided by the results of SDS-PAGE analyses performed by Wright (2009). This study indicated the presence of major toxin bands in the venom of *P. lineatus*, at 15–16 kDa and 13–14 kDa, in addition to the conserved 110 kDa putative toxin found in the venoms of most catfishes. The larger peptide is likely to represent toxin-PC, which showed a similar molecular weight in previous characterizations by Auddy and Gomes (1996) in the related species *P. canius*. The smaller peptide, however, is very similar in molecular weight to the toxic fraction isolated from epidermal secretions of *P. lineatus* by Shiomi et al. (1987, 1988). Wright (2009) also identified a ~39 kDa putative toxin in the electrophoretic profile of the venom of *Arius jordani*. This corresponds closely with the major toxic factor of the skin secretion of the congeneric *A. bilineatus*, which has been isolated and shown to have a molecular weight of approximately 39 kDa (Thomson et al. 1998).

Two scenarios have been proposed to explain the evolutionary origins and derivation of venom glands and their products in catfishes, both of which theorize that these structures and their products are derived from epidermal secretory cells. The first of these, developed by Cameron and Endean (1973) and outlined in the above discussion of siluriform venom gland cellular morphology, hypothesizes that

the venom glands of all fishes, including catfish species, are derived from crinotoxin-producing epidermal cells. In this scenario, the early stages of venom gland development would consist of a thickening of crinotoxin-producing epidermal tissue surrounding the spines of early venomous catfish species, offering a selective advantage to these individuals in deterring potential predators. Subsequent evolutionary changes, including further increased concentrations of toxic protein-secreting cells and their segregation from the epidermal tissue by an integumentary sheath; the suppression of other epidermal cell types from being produced within this tissue; and the movement of this glandular tissue closer to the fin spines, thereby achieving efficient delivery of cellular products during envenomations, then established the morphology of siluriform venom glands as they are seen today. Modifications to fin spines facilitating the delivery of venom into wounds, such as the grooves found in the spines of akysid, amblycipitid, and some ictalurid catfishes (Wright 2009, 2012a, b; Egge and Simons 2011), are hypothesized to have occurred secondarily to the development of venom glands in ancestral species.

It is true that crinotoxins are released when epidermal cells are damaged during predation attempts on catfishes, which is evocative of the manner in which venom is released when the spines of a catfish enter a potential predator. The actual function of these toxins appears to be in the deterrence of fouling organisms, however, as ichthyocrinotoxic species are characteristically sedentary and possess decreased or absent squammation (Cameron and Endean 1973). Crinotoxins have also never been shown to have any appreciable predator deterrent effects, and, in fact, predatory species will readily attack and feed on damaged and distressed catfishes (Bosher et al. 2006; Emmett and Cochran 2010; Wright 2012a) as well as baits coated with stress-related epidermal secretions (Al-Hassan et al. 1985). Studies of the skin secretions of several *Arius* species have indicated that compounds contained therein are able to accelerate healing of wounds and may also have antimicrobial properties (Al-Hassan et al. 1983, 1985, 1987; Robinette et al. 1998). Antimicrobial capabilities have also been demonstrated from the axillary gland secretions of callichthyid catfish species, which are also thought to be derived from epidermal stress-related secretions (Greven et al. 2006; Kiehl et al. 2006), suggesting that secretory cells producing antimicrobial products have already been co-opted into other siluriform secretory structures.

This information led Wright (2009) to propose an alternative selective scenario to the one proposed by Cameron and Endean, centering on the apparent healing and antimicrobial properties of catfish epidermal secretions (see also ▶ Chap. 11, "Venom as a Component of External Immune Defense in Hymenoptera," this volume). The epidermal tissue covering the spines of catfishes is frequently damaged during interactions with predators and with their physical environment. It is therefore conceivable that higher concentrations of epidermal secretory cells surrounding the spine could confer a selective advantage, through improved healing times and decreased opportunities for infection of compromised tissues. This selection would lead to increased aggregations of these cells around the fin spines, with the toxic, antipredatory effects of their secretions being either an epiphenomenon to their primary healing benefits in catfish species or secondarily developed to augment existing defensive structures.

Once venomous secretions had been established and associated with their delivery devices, lineage-specific selective regimes could then act on venom toxicity and composition in catfishes to produce the compositional and toxicological variation found in the venoms of modern siluriform species as well as the conservation of the primary, pain-producing peptides that form the basis of their predator deterrent abilities.

Conclusion and Future Directions

The evolutionary study of siluriform venom glands and their products represents an important but understudied area of inquiry, due both to their human impacts and potential untapped benefits as well as the fact that they represent an important antipredatory trait in a globally ubiquitous group of organisms, which often represent a significant portion of a given region's aquatic vertebrate biodiversity. Though these structures have been shown to provide a formidable defense against predators in several cases, even influencing other aspects of morphological evolution in some genera, secondary losses of venom glands are evident in several groups, most likely due to relaxation of predation pressures due to different aspects of life history and habitat choice. Venomous catfishes comprise a highly diverse group of organisms, possibly outnumbering all other venomous vertebrates combined, and display a correspondingly high degree of variation in venom delivery apparatus morphology and venom effects. In natural predators and laboratory organisms, the venoms of catfishes have been shown to elicit symptoms consistent with cardiovascular, neurotoxic, hemolytic, and/or lethal effects, with a high degree of taxonomic variation in the suite of effects induced by different species' venoms. Despite this, siluriform venoms appear to be quite simplified, consisting of only a few toxic venom proteins per species. Very few siluriform venoms have been studied in any detail, however, and future examinations of inter- and intrafamilial variation in venom toxicity and composition would have great potential to uncover additional venom toxin diversity within catfishes, as well as to generate insights into ecological differences influencing species-specific venom characteristics.

Venom glands have arisen independently at least twice within the order Siluriformes, with the potential for a third origin in the South American family Doradidae. Additional higher-level analyses of siluriform phylogeny are also required to provide greater resolution of basal siluroid relationships and a well-supported consensus of internal relationships within this suborder, which will allow stronger conclusions regarding the developmental history of catfish venom glands to be drawn. Histological, toxicological, and electrophoretic evidence all suggest that the venom glands of catfishes are evolutionarily derived from epidermal secretory cells. Whether catfish venoms are derived from crinotoxins or healing and antimicrobial substances produced by epidermal cells in the skin is unclear, however. Further studies of both epidermal secretion types and the venoms of catfishes are required at a proteomic and genetic level in order to determine the relationships between these different substances. It is entirely possible that these crinotoxins and

antimicrobial agents are one and the same, leaving little hope for possible resolution to the debate regarding which defensive selective force, increased predator deterrence, or rapid healing and infection defense initiated the process of venom evolution in catfishes.

Studies making use of next-generation proteomic, transcriptomic, and genomic technologies and analyses have great potential to generate desperately needed data regarding the identity and structure of siluriform venom toxins, their physiological targets, their genetic origins, and the selective forces driving their evolution. Continued studies of catfish venoms have the potential to greatly increase our understanding of the general ecology and evolution of this hyperdiverse order of fishes as well as to generate insights into the evolution of venoms as defensive traits. The chemical complexity of the venoms of species utilizing these secretions in prey capture makes it exceedingly difficult to determine whether and how selection for prey capture or predator defense has influenced any particular venom component, as these differing selective forces have the potential to be non-complimentary. The relatively simple composition of catfish venoms, and fishes in general, as well as their use in a strictly defensive capacity, therefore presents an outstanding opportunity to study the selective factors influencing defensive venom evolution, examinations of which are largely absent from the literature.

Cross-References

► Venom as a Component of External Immune Defense in Hymenoptera

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Evolution of the Snake Venom Delivery System

15

Harald M. I. Kerkkamp, Nicholas R. Casewell, and Freek J. Vonk

Abstract

There are over 3,000 species of snakes known to man. These limbless predators have been divided into two groups, the basal snakes (Henophidia) and the advanced snakes (Caenophidia). Venom evolved prior to the advanced snake radiation and, consequently, many use venom to subdue their prey. To do so, venom is injected via the use of a venom delivery system. The venom delivery system includes a postorbital venom gland on each side of the upper jaw that is associated with specialized venom-conducting fangs or teeth. Both the venom gland and fangs are considered to have originated from a common ancestor and are thought to be developmentally linked to one another. Even though the venom gland has a common ancestral origin, it can exhibit considerable morphological variation among the main snake families. Similarly, the fangs can occupy various positions on the upper jaw but are always found on the maxilla. Caenophidians are often referred to by the position of their fangs as either rear- or front-fanged snakes. The vast majority of snakes that are medically important to humans are front-fanged, and this character has evolved independently on at least three occasions. In addition, some front-fanged snakes have evolved a secondary

H.M.I. Kerkkamp (✉)
Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Leiden, The Netherlands
e-mail: h.m.i.kerkkamp@biology.leidenuniv.nl

N.R. Casewell
Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool, UK
e-mail: nicholas.casewell@lstmed.ac.uk

F.J. Vonk
Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Leiden, The Netherlands

Naturalis Biodiversity Center, Leiden, The Netherlands

Molecular Ecology and Evolution Group, School of Biological Sciences, Bangor University, Bangor, UK
e-mail: freek.vonk@naturalis.nl

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_11

gland associated with the venom system, known as the accessory gland. The venom glands, accessory glands, and fangs of different caenophidian snake families exhibit substantial morphological differences reflecting their evolutionary history. However, further studies are required to fully elucidate the ecological significance of differences in fang position, the function of the accessory gland, and the driving forces underpinning the convergent evolution observed in the snake venom delivery system.

Keywords

Venom delivery systems • Evolution • Advanced snakes • Venom glands • Accessory gland • Front-, rear-fang

Contents

Introduction

Snakes have been both feared and revered by humans throughout the ages, having been a symbol to the gods in Mayan culture and a symbol of evil in Christian faith. Indeed, these venomous animals have left, with their frequently lethal encounters (Kasturiratne et al. 2008), an everlasting impression on humans. It is even believed that they might have played a prominent part in the evolution of the primate brain and sensory system (Isbell 2006). Snakes themselves are limbless tetrapods that are represented on earth today by some 3,150 species (Vidal et al. 2007; Vonk et al. 2011); they are highly specialized predators and they inhabit most major ecosystems. All snakes share a unique body plan having undergone an elongation of the body, a loss of most clear morphological boundaries, and a substantial increase of the number of vertebrae (up to 500) (Vonk and Richardson 2008). Though their different body plan gives snakes a distinctive appearance, it is the incongruity between their often fragile appearance and the devastating damage they can inflict with their venom that is notable. Whereas the Henophidia (basal snakes such as pythons and boas) rely on constriction for prey capture, the vast majority (ca. 2,700 species) of snakes utilize venom. This large group, known as the Caenophidia (advanced snakes), represents a single massive diversification event that occurred after the Cretaceous-Tertiary boundary and the extinction of the dinosaurs around 64 million

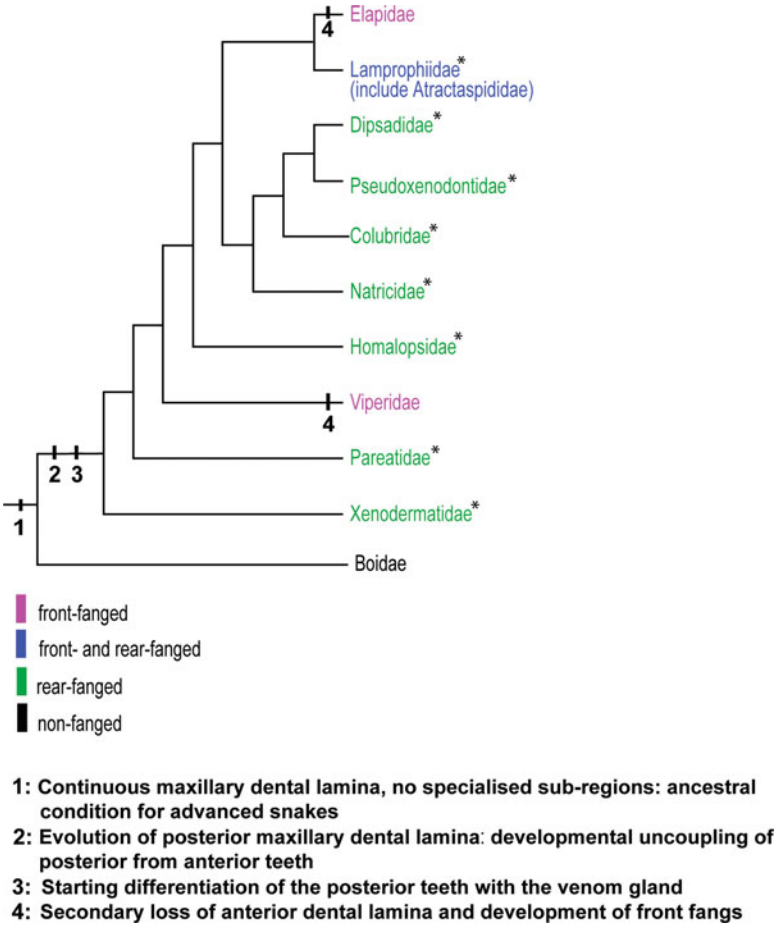

Fig. 1 Phylogeny from Vonk et al. 2008. The evolutionary changes leading from an unmodified maxillary dentition to the different fang types in advanced snakes are indicated at the nodes: (1) continuous maxillary dental lamina, no specialized subregions – ancestral condition for advanced snakes; (2) evolution of posterior maxillary dental lamina – developmental uncoupling of posterior from anterior teeth; (3) starting differentiation of the posterior teeth with the venom gland; (4) loss of anterior dental lamina and development of front fangs. *Asterisks* indicate the families represented by the traditional "colubrid" name

years ago (Vidal et al. 2009; Vonk et al. 2011). Most of the advanced snakes (Caenophidia) have the ability to inject venom into prey using their venom delivery system (Warrell 2010). The major snake families within the Caenophidia are the Viperidae (e.g., vipers, adders, pit vipers, and mocassins), the Elapidae (e.g., cobras, mambas, kraits, coral snakes, taipans, and sea snakes), the Atractaspididae (burrowing asps), and the Colubridae sensu lato (Fig. 1). The term Colubridae sensu lato represents a traditional name for all snakes with a venom gland whose venom poses no danger to humans (i.e., any species of advanced snake that is not a

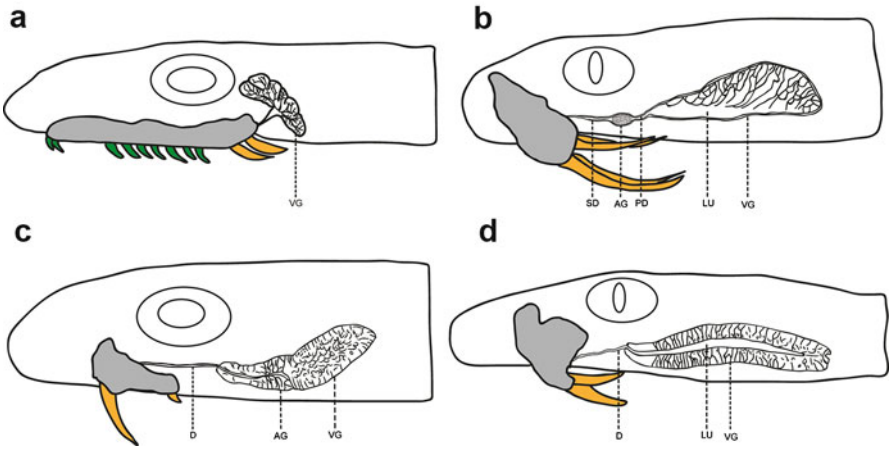

Fig. 2 Schematic ventral views of adult skull and in situ of venom delivery system of (**a**) "colubrids,"(**b**) Viperidae, (**c**) Elapidae, and (**d**) Atractaspidinae. Maxilla colored *gray*, teeth colored *green*, fangs colored *orange*, *AG* accessory gland, *D* duct, *SD* secondary duct, *PD* primary duct, *LU* lumen, *VG* venom gland (Redrawn from Kochva (1987) and Vonk et al. (2008))

member of the Viperidae, Elapidae, or Atractaspididae). Recently, the Colubridae have been shown to be paraphyletic and have been redefined with many subfamilies (e.g., Dipsadidae, Pseudoxenodontidae, Colubridae, Natricidae, Lamprophiidae, Homalopsidae, Pareatidae, and Xenodermatidae) being elevated to a family level to reflect their evolutionary distinctiveness (Fig. 1) (Vidal et al. 2007; Lawson et al. 2005). However, for clarity of description, the traditional "colubrid" name will be resurrected to represent these families here.

While venom delivery systems have evolved independently among vertebrates on multiple occasions (Casewell et al. 2013), advanced snakes exhibit the most complex, specialized, and variable venom system. Its main function is the production of a specialized toxic secretion in the venom gland and its delivery from this gland into prey (and occasionally predators or aggressors, such as humans). The venom delivery system of advanced snakes primarily consists of a postorbital venom gland associated with specialized venom-conducting fangs (Fig. 2). Though the fangs of advanced snakes can occupy various positions on the upper jaw (e.g., at the caudal or rostral end), they are always found on the maxilla (Vonk et al. 2008). In this chapter, the components that comprise the snake venom delivery system will be discussed. First, the venom itself will be examined as a secretion, before discussing the venom-producing glands, the venom ducts, the fangs, and the specialized accessory gland found downstream of the venom gland in some species (Fig. 2). Second, a comparative analysis of the development of the venom glands and fangs for each of the advanced snake families will be provided and, finally, an overview of the current understanding of the evolution of the venom system of advanced snakes.

Venom

As limbless predators, the majority of advanced snakes rely on venom to immobilize or incapacitate their prey. Snake venom is a complex cocktail of bioactive components, largely consisting of a mixture of proteins and peptides (referred to as toxins) but also containing salts and organic compounds such as amino acids and neurotransmitters. Venom toxins are biologically active proteins most of which are encoded by several multilocus gene families, which function synergistically to facilitate prey incapacitation and capture (Casewell et al. 2013; Vonk et al. 2013). A few venom toxins appear to be modified salivary gland secretions, whereas many venom genes have originated from housekeeping genes co-expressed in other organs, which have either been recruited for, or restricted to, expression in the venom gland (Fry 2005; Vonk et al. 2013; Hargreaves et al. 2014; Junqueira-de-Azevado et al. 2015; Reyes-Velasco et al. 2015). Following their recruitment to the venom gland, it is apparent that gene duplication events are critical for the expansion of the toxin repertoire found in the venom of many of the advanced snakes (e.g., Casewell et al. 2011, 2014; Vonk et al. 2013; Sunagar et al. 2013). Once injected, venom toxins permeate from tissue and become systemic via dispersal by the bloodstream and lymphatics. Venom toxins are capable of interacting with a wide variety of physiological proteins and receptors present in their prey, ultimately resulting in disruptions to the central and peripheral nervous system, the blood coagulation cascade, the cardiovascular and neuromuscular system, and/or the homeostasis in general. Synergism between different venom toxins also appears to be commonplace, with distinct or related proteins present in venom capable of targeting multiple different steps of the same physiological pathway, such as the coagulation cascade or neurotransmission.

Venom Glandular Apparatus

The venom glandular apparatus is a compilation of all the components utilized by advanced snakes to produce, store, and deliver venom to the fangs. Though there is a high degree of variability in the apparatus components, all advanced snakes possess a pair of postorbital glands in which venom is secreted and stored. These structures are naturally referred to as venom glands. The glands are enclosed in a fibrous sheath that, in some species, permits the attachment of muscles. These venom glands are considered to be specialized postorbital oral glands that produce venom and are developmentally coupled to fang formation (Kochva and Gans 1965). Through morphological, embryological, and developmental biological studies, the venom glands found in the different caenophidian snake families are considered to be homologous and therefore to be the result of a single origin at the base of the colubroid radiation between 60 and 80 million years ago (Fry 2005).

From this early common origin of the venom gland, the venom glandular apparatus has undergone severe evolutionary tinkering in each of the Colubridae,

Viperidae, Elapidae, and Atractaspidinae snake families. In "colubrids," the venom gland is positioned posterior to the eye, usually encased in a thin cover of connective tissue, and consists mostly of serous cells (i.e., specialized to secrete an enzyme solution). A single, short duct extends anteromedially from the lumen of the gland to the base of the posterior fang (Fig. 2a). In contrast, the viperid venom gland is large and generally triangular in shape, with the longest side of the triangle found along the upper lip and the rounded apex directed dorsally. The main venom gland has a complex tubular structure and is divided into several lobules by infoldings of the outer sheath. The lumen is voluminous and can store a large quantity of venom. Anteriorly, the triangle comes to a point as the lumen forms the primary venom duct. This duct then passes through an accessory gland, leaving it as the secondary duct, which extends to the sheath of the fangs. Just proximal to the accessory gland, the primary duct narrows, forming a glandular isthmus, which restricts the passage of venom to the distal components of the venom gland apparatus (Fig. 2b) (Mackessy and Baxter 2006). The elapid venom gland is oval in shape. It is made up from a main venom gland and an accessory gland, but, in contrast to vipers, does not have a primary duct connecting the two. Instead, the accessory gland forms the distal part of the venom gland. The main venom gland is made up of many simple or branching tubules. The lumen is narrow and most of the venom is stored within secretory cells rather than the lumen. The accessory gland surrounds the entire duct, so that the narrow lumen continues through it (Fig. 2c). The atractaspid venom gland is cylindrical. A central lumen extends along the length of the gland with a characteristic pattern of unbranched tubules radiating outwards. There is no distinctive accessory gland, but mucous cells line the lumen along most of the length of the gland, in contrast to the serous cell lining observed in the vipers and elapids (Fig. 2d) (Jackson 2002).

Venom Gland

Venom glands reside next to the upper jaw behind the eye, although in some taxa they can be highly elongated, extending posteriorly along the body well beyond the head (Fry et al. 2008). The main venom gland contains at least four distinct cell types: secretory cells (79%), mitochondria-rich cells (2%), horizontal cells (10%), and dark cells (9%) (Mackessy 1991). Venom glands are innervated by the maxillary branch of the trigeminal nerve (V2) with contributions from the facial nerve (VII) (Kochva 1965; Taub 1966), and their vascular supply is from branches of the internal carotid artery (Phisalix 1922; Kochva 1965). Depletion of stored venom stimulates a new cycle of venom synthesis, and the secretory epithelium undergoes morphological and biochemical changes. In response, the epithelial cells change their shape from cuboid to columnar; the cistern of the rough endoplasmic reticulum expands and venom is synthesized (Carneiro et al. 1991; Kochva et al. 1980; Mackessy 1991; Warshawsky et al. 1973). This happens in response to stimulation by the autonomic nervous system (Kerchove et al. 2004; Yamanouye et al. 1997). The venom production cycle lasts around 30–50 days and, within 4–8 days, proliferation of the rough

endoplasmic reticulum and messenger ribonucleic acid (mRNA) levels have reached their peak (Carneiro et al. 1991; Kochva et al. 1980; Mackessy 1991; Rotenberg et al. 1971; Yamanouye et al. 1997), and subsequent merocrine exocytosis results in the replenishment of venom in the epithelial ductules and large basal lumen. Completion of the synthesis and secretion stage occurs approximately 16 days after depletion of the gland, and during this period, cells cycle from cuboidal to columnar and back to cuboidal morphology (Kochva 1987; Kochva et al. 1975; Mackessy 1991). The venom is then stored in the basal lumen and ductules of the venom gland for varying periods of time and is therefore available when needed (Mackessy and Baxter 2006).

Compressor Musculature

As previously described, the venom glands are enclosed in a fibrous sheath that, in some snakes, facilitates the attachment of muscles. The musculaturization of the venom glands observed in Viperidae, Elapidae, and Atractaspidinae snakes allows the ejection of venom from the glands and injection into prey in a high-pressure manner (Vonk et al. 2011). The contracting muscle that compresses the venom gland is the muscularis compressor glandulae (Rosenberg 1967; Jackson 2007; Warrell 2010). Though all three families possess this musculature, they are not homologous. The elapid compressor glandulae muscle is derived from the adductor externus superficialis muscle, the viperid from the adductor externus profundus muscle, and the atractaspididae from the adductor externus medialis muscle (Jackson 2007).

Accessory Gland

The accessory gland, a glandular structure found in Viperidae and Elapidae snakes, is associated with the main venom gland and has two distinct regions: the anterior and posterior. The anterior region has a simple secretory epithelium with at least six types of cells: two types of secretory cells, mitochondria-rich cells without secretion, horizontal cells, dark cells, and basal cells. The posterior region has a simple epithelium with two types of cells: seromucous cells and horizontal cells (Sakai et al. 2012). In elapids, the accessory gland begins immediately anterior to the main gland and completely surrounds the entire duct of the venom gland. In viperids, the accessory gland is considerably smaller and surrounds the anterior end of a primary duct (Kochva and Gans 1970). As in the main venom gland, the anterior region of the accessory gland displays a long secretion production cycle lasting around 15 days. The peak of secretion occurs 4 days after venom extraction, and cells are replenished with secretory vesicles approximately 15 days after venom extraction (Sakai et al. 2012).

The primary function of the accessory gland remains unknown. It has been postulated that it may condition or activate venom passing through it during injection (Gans and Elliott 1968). The presence of serous cells caudally, followed rostrally by

mucus-secreting epithelium (Hattingh et al. 1984; Mackessy 1991), implies that lytic venom components passing through the accessory gland are activated by the caudal portion (Mackessy and Baxter 2006). The accessory gland, especially the rostral part, may actually contribute substances to the venom during injection. Supporting this hypothesis is evidence that the accessory gland appears to express most toxins that are expressed in the venom gland, although it does so at a much lower level (Vonk et al. 2013). However, electrophoresis and reverse-phase high-performance liquid chromatography analyses have yet to identify any peptide or protein components that have been added to the venom bolus exiting the intact apparatus, when compared with samples taken extracted from the main venom gland (Mackessy and Baxter 2006; Weinstein et al. 2010).

Fangs

Fangs are grooved or hollow teeth used to deliver venom. There is extensive variation in both the morphology and the position of the fangs throughout the advanced snakes, but the fangs are always located on the maxilla. In addition, the length of the maxilla is also variable. For example, in viperids and atractaspids, it is simply a stub serving as a base for the fang, whereas in "colubrids" the maxilla is long and forms the base for teeth in addition to the fangs. Like all teeth, fangs are continuously replaced throughout the life of the snake. The functional fang is ankylosed to the maxilla, with a series of replacement fangs, which are not attached to any dentigerous bone, caudal to it. These replacement fangs are formed starting with the distal tip and growing by gradual accretion of material to the proximal end. When the functional fang is shed, the replacement fang will ankylose to the maxilla and take its place (Jackson 2007; Zahradnicek et al. 2008).

Snakes can be divided into three groups based on their fang position: (i) snakes with no fangs, (ii) rear-fanged snakes, and (iii) front-fanged snakes. The Henophidia have no fangs and instead only have teeth on the maxilla, the "colubrids" are rear-fanged snakes, and the Viperidae, Elapidae, and Atractaspidinae are front-fanged snakes. Unfanged snakes have no specialized teeth on the maxilla. The maxillary teeth develop from a single continuous maxillary odontogenic band, and this band invaginates to form one dental laminae, from which all teeth develop.

In rear-fanged snakes, the fangs are positioned at the caudal end of the maxilla, whereas there are normal teeth present on the rostral end of the maxilla. The fangs can be ungrooved and resemble enlarged teeth (with some slight morphological differences) or grooved to facilitate venom delivery by possessing an open channel along the lateral or anterolateral surface (Jackson 2007). The maxillary teeth and fangs of the rear-fanged snakes develop from two dental laminae which invaginate separately and fuse during development. The anterior and posterior dentitions have been found to be developmentally independent from each other: the anterior dental laminae only bear teeth, similar to those found in non-fanged snakes, whereas the posterior laminae bear both teeth and the common primordium with the postorbital gland that develops into both the rear fangs and the venom gland (Vonk et al. 2008).

In the front-fanged snake families, the fang is positioned at the rostral end of the maxilla, and there are no normal teeth present. The front-fangs are more or less closed, hypodermic-like, tubular venom-injecting fangs that are open through two orifices situated at the top and bottom on the ventral tooth side. At the fang base, one orifice is connected to the venom duct, and at the apex of the fang, the channel that runs through it does not extend all the way to the distal tip of the fang but instead ends somewhat more proximally to the tip, thereby forming a beveled tip like that of a hypodermic needle (Jackson 2007; Zahradnicek et al. 2008). The maxillary front fangs develop from a single maxillary odontogenic band. This maxillary odontogenic band is found in the posterior part of the upper jaw, and there is no dental lamina in the anterior region of the jaw. The odontogenic band invaginates normally and forms one dental lamina; the fangs develop from the posteriormost part of this lamina, and no other teeth develop on the anterior part of it. The anterior toothless part of the dental lamina has been termed the dental ridge and has been described in both the Viperidae and Elapidae. The fangs then displace from their embryonic posterior position into its adult front position by ontogenetic allometry (Vonk et al. 2008). Front fangs initially develop in a very similar fashion to the rest of the dentition, going through the bud, cap, and bell stages of development. However, at the bell stage, an invagination is observed on the ventral side of the developing tooth. On the ventral side, the epithelial wall of the developing tooth germ invaginates, and epithelial cells invaginate into the dental mesenchyme, and the sides of the invaginating wall make contact and fuse to form an enclosed canal. The epithelial cells proliferate to enlarge the canal and then die by apoptosis, forming an empty tube through which the venom flows. The orifices at each end of the canal develop by a similar invagination, but the initial width of the invagination is different from that in the middle of the tooth, and it is associated with higher proliferation. The two sides of the invaginating epithelium never come into contact, leaving the orifice open. Once the infolding process has occurred, hard tissue is laid down; fangs are formed from the distal tip and grow by gradual accretion of material to the proximal. The fang is thus built up from the tip to the base (Zahradnicek et al. 2008).

Although all three front-fanged families share these tubular fangs, elapid, viperid, and atractaspidid, snakes are not monophyletic and, consequently, their fangs exhibit some differences. Similar to "colubrids" the Elapidae have a fixed maxilla to which the front fangs are attached, whereas the Viperidae have front fangs that are mounted on moveable maxilla that allows the fang to be erected when biting and remain parallel to the jaw when relaxed. Similarly, the Atractaspidinae have moveable maxillae with fangs attached (Jackson 2007; Zahradnicek et al. 2008). In addition, in viperids the surface of the anterior side of the fang between the entrance and discharge orifices is smooth, whereas elapids and atractaspids have an anterior suture line connecting the two orifices, although the venom canal is still enclosed (Jackson 2007; Zahradnicek et al. 2008).

A further adaptation of the fangs is seen within the Elapidae family, where the fangs of some cobras (genus *Naja*) have been modified such that they are capable of spitting their venom toward an aggressor, to a distance of at least 3 m. While most elapid snakes have a fang exit orifice appearing as an elongated/hypodermic-like

opening, the exit orifice of spitting cobras is directed more cranial and is more or less tear shaped in outline with the lower edge of the opening appearing rounded, while the upper end of the opening terminates where the walls of the venom canal meet the suture (Young et al. 2004; Westhoff et al. 2005; Bogert et al. 1943). This fang morphology has been observed in members of both the African and Asian cobras, and the spitting cobras are therefore not monophyletic (Wüster et al. 2007). In addition, the African rinkhals (*Hemachatus haemachatus*), an elapid near relative of the cobras, has also developed the ability to spit its venom. Spitting is therefore considered to have arisen several times independently (on at least three occasions) in the elapid family (Young et al. 2004; Westhoff et al. 2005; Wüster et al. 2007).

View on Evolution

It is thought that the venom delivery system of all advanced snakes shares a common ancestral origin (Fry et al. 2008) (Fig. 1). This ancestor likely possessed a single continuous maxillary dental lamina with no specialized subregions and a mucous secreting post orbital gland from which the ancestral venom delivery system evolved. It was previously hypothesized that the snake venom gland evolved by evolutionary modification of the pancreatic system (Kochva 1987). While this hypothesis has not been borne out (e.g., the toxins found produced in the snake venom gland do not appear to be predominately from a pancreatic origin Fry 2005; Hargreaves et al. 2014; Junqueira-de-Azevedo et al. 2015; Reyes-Velasco et al. 2015), a recent analysis of micro-ribonucleic acid (miRNA) libraries of the venom gland showed molecular similarities between venom gland miRNAs and those previously identified from the human and mouse pancreas (Vonk et al. 2013). These results suggest that certain components of a core genetic network regulating secretion may have been co-opted from an ancestral role in the pancreas during the evolution of the snake venom gland (Vonk et al. 2013). Regarding fangs, it is believed that at the base of the advanced snakes, a posterior subregion of tooth-forming epithelium became developmentally uncoupled from the remaining dentition and has become developmentally linked to the primordium of the venom gland. This has allowed the posterior teeth to develop in close association with the venom gland and independently from the rest of the maxillary teeth. Subsequently, the posterior maxillary dental lamina became fang bearing and the venom gland became protein secreting, resulting in the first venom delivery system. Taking into account the structural homologies observed in front-fanged snakes, extant rear-fanged snakes appear to represent the venom system most closely related to the ancestral state. For example, derived characters observed in front-fanged snakes include the toothless dental ridge (observed in elapids and viperids), which is similar to the part of the posterior dental lamina that fuses with the anterior dental lamina in rear-fanged snakes. In addition, the posterior developmental origin of the front fangs in both elapids and viperids, along with the invagination mechanisms by which the orifices in the front fang are formed, are similar to the grooving of the rear fangs (Vonk et al. 2008; Zahradnicek et al. 2008). It therefore appears that, from the ancestral state, the

venom delivery system has evolved tubular anterior fangs with an associated muscularized venom gland in at least three different lineages (viperids, elapids, and atractaspids) (Vonk et al. 2008; Chipman 2009). It is notable that these three lineages contain the vast majority of venomous snakes that are known to be medically important to humans. The muscularized front-fang system facilitates the rapid injection of voluminous venom absent in most rear-fanged snakes. In addition, it is apparent that two unrelated lineages (viperids and elapids) have independently evolved an accessory gland downstream of the venom gland, but it remains unclear what the function of these glands are in these families.

Conclusion and Future Directions

Snakes are vertebrates that have undergone a number of notable adaptations, from their loss of limbs and elongation of their body plan, to the physiological extremes associated with digesting large intact prey items (Di Poi et al. 2010; Castoe et al. 2013). However, it is the venom delivery system which is perhaps their most renowned adaptation, and it represents one of the most sophisticated weapon systems found in the animal kingdom. The main components of this system are the venom-secreting gland and the venom-conducting fangs (Fig. 2). Interestingly, the front-fanged system of viperid, elapid, and atractaspid snakes appears to have evolved by the process of convergent evolution. Convergent processes also appear to underlie the fang adaptations observed in the spitting cobras.

The driving forces underlying the convergent adaptations observed in the snake venom system remain predominately unknown. Therefore, it is apparent that to better understand the evolutionary mechanisms acting on the venom delivery system, we need additional information. For example, the ecological significance of front versus rear fangs remains ambiguous. It is highly plausible that selection would favor tooth morphologies that are more efficient at introducing venom into prey, such as grooved over ungrooved fangs or fangs with a closed channel over deeply grooved fangs. Furthermore, whereas rear-fanged snakes utilize a bite-and-hold method of prey capture, front-fanged systems appear to have enabled certain snakes to feed on larger (and potentially more dangerous) prey through a bite-and-release method of venom injection – this adaptation may therefore have allowed those snakes to occupy different niches to many rear-fanged snakes. In summary, it is plausible that the front-fang venom systems have evolved convergently because of increased efficiencies, or in response to different lineages exploiting new predator niches in a similar manner, or perhaps simply because the existing fang developmental pathways allow limited adaptation (Zahradnicek et al. 2008).

Secondly, the venom system of elapids and viperids both contain an accessory gland (Gans and Kochva 1965; Mackessy 1991; Mackessy and Baxter 2006) (Fig. 2). The function of this small enigmatic gland in these different lineages remains unclear. It has been speculated that accessory gland secretions keep venom from continuously draining into the mouth of the snake, that they facilitate the venom flow through the fang (Gans and Kochva 1965), or are a source of toxins,

or that they function to activate the toxins of the venom gland (Gennaro et al. 2007). The conserved morphology of the accessory glands and the many cell types present do suggest that they possess an important functional role (Mackessy and Baxter 2006). However, considering that the accessory gland is only found in the Elapidae and the Viperidae, which do not form a monophyletic group (Fig. 1), it remains unclear whether these accessory glands are homologous, share a similar function, and have been lost from other advanced snakes over evolutionary time, or whether they have evolved independently in elapids and vipers and therefore potentially function in a differential manner.

Through the application of a variety of new molecular, genetic, and genomic methods available to scientists today, it is possible that these fundamental questions can be answered to shed light on the evolutionary mechanisms that underlie the fascinating diversity of venom delivery systems observed in snakes.

Cross-References

- ▶ A Critique of the Toxicoferan Hypothesis
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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Part IV

Systematics of Venomous Groups

Systematics of Siphonophores

16

Gillian M. Mapstone

Abstract

Siphonophores are the most complex of all pelagic medusozoan hydrozoan cnidarians, bearing various types of zooids on a long stem and often termed "string jellyfish." They are extremely fragile and live almost exclusively in the open ocean. They vary in length from 50 m down to 10–20 mm. Most species bear swimming bells (nectophores) for locomotion, some have a float (pneumatophore), and all have a long stem of iterative units termed cormidia for feeding, reproduction, and also protection and buoyancy. Tentacles from the cormidia bear stinging cells (nematocysts) for prey capture, either in simple groups or lines or in more complex nematocyst batteries on side branches known as tentilla. In life, tentacles and their side branches extend into a three-dimensional net for fishing, into which prey either blunders by accident or, in a few species, is attracted by lures. Such great diversity has led to a complex systematics based on a range of morphological characters, recently enhanced by the first molecular study of the group. From this a new phylogeny has been proposed, for 17 valid families (one semi-benthic) and 177 valid species (some unassigned). Characters of these families are reviewed in two tables and 17 summaries, including diagnostic characters, number and variety of species, and, where appropriate, habitat preferences and relative success in today's seas. Figures and images showing different types of siphonophores, their morphology, stinging organs, and appearance in life accompany the main text.

Keywords

Siphonophores • Cystonects • Physonects • Calycophorans • Hydrozoa • Nematocysts • Nectophores • Cormidia, pneumatophore • Tentacles • Eudoxid

G.M. Mapstone (✉)
Department of Life Sciences, The Natural History Museum, London, UK
e-mail: g.mapstone@nhm.ac.uk

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_14

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Introduction

Siphonophores are complex pelagic cnidarians in the medusozoan group Hydrozoa. They are carnivorous "sit and wait" predators, some of which can lure prey into their tentacles by mimicking shoals of small copepods, medusae, or fish. They rapidly stun their prey with toxins delivered from batteries of nematocysts on the tentacles or tentacle side branches. This is essential because, otherwise, the siphonophore itself would be damaged by struggling prey attempting to escape.

Individuals possess enormous powers of extension, contracting their stem and tentacles completely for swimming and then relaxing them to the maximum for feeding. This relaxation allows formation of an enormous three-dimensional fishing net, or web, often with the aid of swirling swimming movements to "set the trap." Once set, many siphonophores simply remain stationary in the water until prey blunders into their net. Others may lure them in either by drawing copepod-like stinging batteries through the water or by flicking red lures resembling shoaling fish to attract prey.

Cnidaria are an ancient lineage, characterized by the presence of cnidae, or stinging cells, most of which are nematocysts. In siphonophores, nematocysts are grouped into either pads on the tentacles (e.g., *Physalia*, the Portuguese man-of-war) or, in most other species, complex nematocyst batteries on side branches from the tentacles termed tentilla.

Siphonophores are often called string jellyfish, or chain jellyfish, to distinguish them from true jellyfish and hydromedusae, which are mostly disk shaped. All belong to one of two major clades which comprise the Cnidaria, namely, the Medusozoa. This clade includes Hydrozoa, Scyphozoa, Cubozoa, and Staurozoa. Most jellyfish in these groups have a bottom-living stage in their life cycle that restricts them to the shelves around most continents. Siphonophora, however, are holopelagic (except one family), meaning that they pass through their entire life cycle in the water column, without having a benthic stage. This has enabled them to penetrate all oceans, and most exhibit a worldwide, or cosmopolitan, distribution. Some species are restricted to tropical latitudes, others to temperate latitudes, and a few are truly cosmopolitan, with records from all around the globe from Arctic to Antarctic waters (Mapstone 2014, Tables 2 and 3).

Siphonophore specimens are difficult to obtain, because they inhabit the deep sea, and are therefore absent from net catches used to monitor coastal waters. Today most are collected by either blue-water SCUBA (self-contained underwater breathing apparatus) or remotely operated vehicles (ROVs), but in the past when nets were used, specimens were often fragmented and damaged and the importance of siphonophores in pelagic assemblages not fully appreciated. Gelatinous animals have traditionally been preserved in buffered formaldehyde to preserve their shape, and vast collections are present at a few locations around the world, including the Natural History Museum, London, but these are of little use for molecular work, which requires alcohol-preserved material.

Siphonophores are colonial polymorphic hydrozoans with physiological integration of zooids and a complex morphology. They also exhibit a diversity of body form. An understanding of their morphology is therefore needed to investigate their systematics, and morphology was very well explained in a seminal monograph of 1965 by A.K. Totton. Little then changed until the first molecular analyses of the group by Dunn et al. (2005b), which revealed some new relationships and diagnostic characters within the group. Characters not previously thought to be important were found to be diagnostic, including the presence or absence of swimming bells, the sexual state of the family or species, the presence or absence of a muscular free zone in the wall of the nectosac of the nectophores, and the type of cormidia present on the stem. These, and other more traditional characters, are reviewed below.

Taxonomic Status

Siphonophora are a small monophyletic group in the phylum Cnidaria and members of the diverse clade Hydrozoa, comprising c. 3,500 species (Fig. 1a). The Hydrozoa includes the large subclass Hydroidolina (3,317 species) and the much smaller subclass Trachylina (155 species) (Fig. 1b). The Siphonophora are a small group of c. 177 species within the Hydroidolina, while the remainder of this subclass comprises the much larger groups Anthoathecata and Leptothecata (c. 3,140 species). These latter two clades are mostly meroplanktonic, with a bottom-living

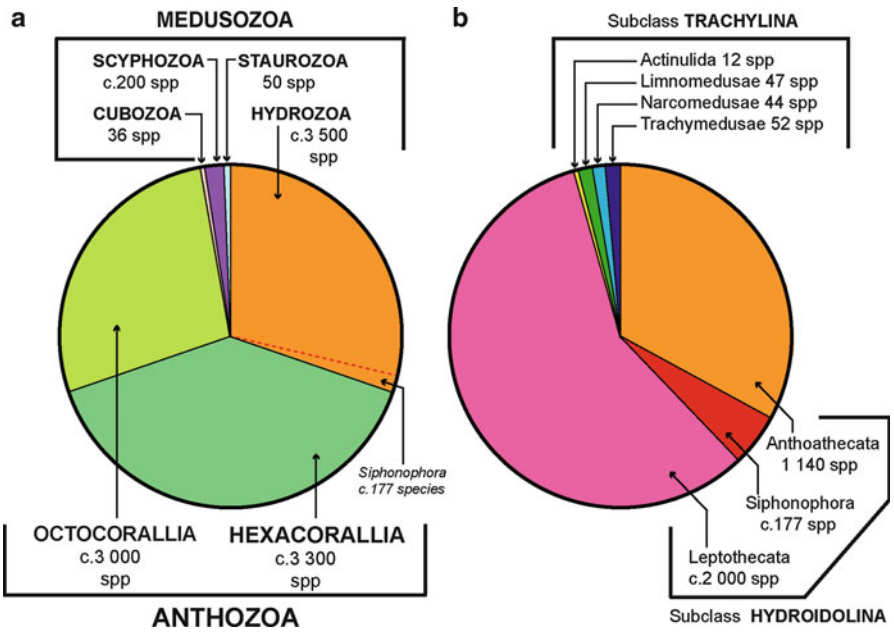

Fig. 1 Position of Siphonophora within the phylum Cnidaria. (**a**) the c. 10,000 Cnidaria species (excluding Myxozoa) subdivided into clades; (**b**) the c. 3,500 Hydrozoan species, subdivided by ranks (Modified from © Gillian Mapstone (2014), Fig. 2A, B; see legend for this figure and Mapstone (2015) for origins of other numbers used in **a** and **b**)

polypoid "hydroid" stage in the life cycle. As noted above, this restricts their geographical distribution and makes them species-rich. Siphonophores, in contrast, are a depauperate species-poor clade and in this respect resemble the other two holopelagic hydrozoan groups Trachymedusae and Narcomedusae, which together comprise c. 96 species (Fig. 1b) (World Hydrozoa Database).

Typical Body Plans and Cormidia

The basic morphology of siphonophores is best explained with reference to the body plans of three typical species from the groups Cystonecta, Physonectae, and Calycophorae (Fig. 2). An anterior-posterior axis related to the direction of swimming is recognized in all siphonophores (reviewed by Mapstone 2009), which in Fig. 2 is oriented vertically, with the anterior end uppermost.

All three types have a long stem, the siphosome, which bears repeating, or iterative, groups of zooids known as cormidia. One cormidium always includes a gastrozooid with tentacle for feeding and one or more sexual zooids for reproduction, often on a tree-like stalk known as a gonodendron. In addition, the cormidia of physonects and calycophorans also include one or more bracts (Fig. 2b, c). A float, the pneumatophore, is present in cystonects and physonects (Fig. 2a, b), and

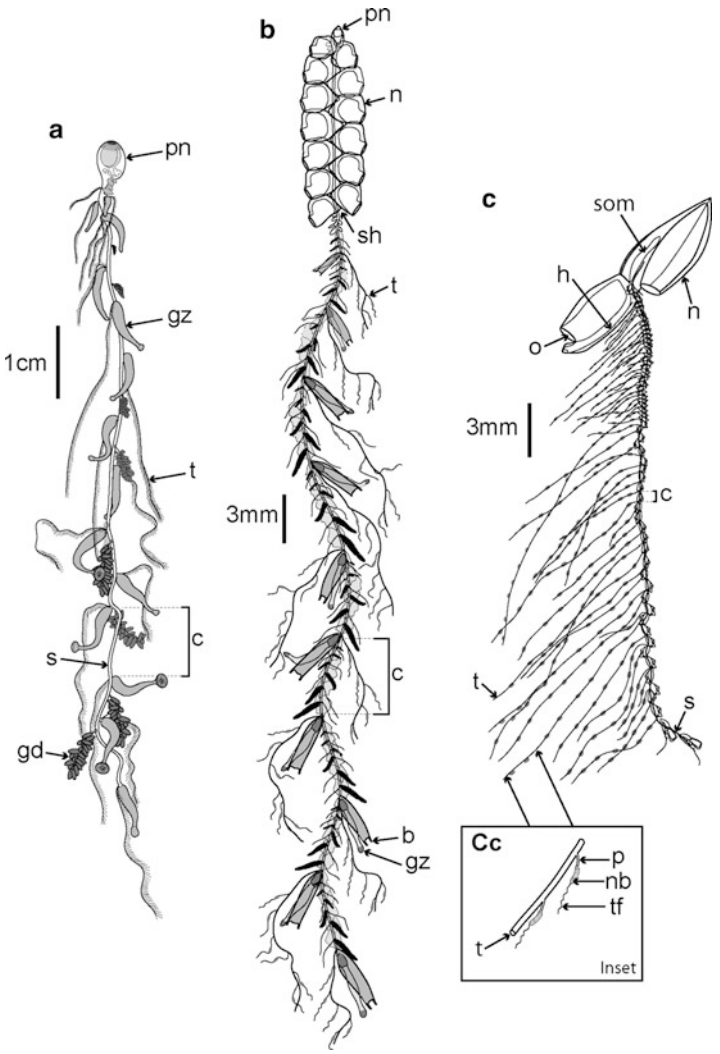

Fig. 2 Siphonophore morphology. Three typical body plans: (**a**) long-stemmed cystonect *Rhizophysa eysenhardti*, (**b**) long-stemmed physonect *Nanomia bijuga*, and (**c**) typical calycophoran *Lensia conoidea*; *inset* **Cc** shows two tentilla attached to one tentacle. Labels: *b* bract, *c* cormidium, *gz* gastrozooid, *gd* gonodendron, *h* hydroecium, *n* nectophore (swimming bell), *nb* nematocyst battery (a cnidoband), *o* ostium, *p* pedicel, *pn* pneumatophore (float), *s* stem, *sh* siphosomal horn (growth or budding zone), *som* somatocyst, *t* tentacle, *tf* terminal filament (Derived from © Gillian Mapstone (2014), Fig. 3; refer to this figure legend for details of published figures and references from which drawings derived)

swimming bells, the nectophores, are present in physonects and calycophorans (Fig. 2b, c). An additional length of stem, the nectosome, carries the nectophores in Fig. 2b (see definition in Mapstone 2009, p. 72).

The pneumatophore is filled with carbon monoxide secreted by a gas gland, and this provides buoyancy for cystonects and physonects. Additional buoyancy is gained from gelatinous tissues in physonects and calycophorans, particularly from bracts in the cormidia; these tissues have a lowered specific gravity from the partial replacement of sulfate ions by chloride ions in the mesoglea (Mackie et al. 1987).

Propulsive zooids, the nectophores, are characteristic of physonects and calycophorans, and each contains a muscular nectosac which undergoes repeated contractions during swimming to facilitate active locomotion through the water (water exits via the ostium). Nectophores are absent in cystonects, which can only drift passively, and, by repeated contraction and expansion of the stem and tentacles, extend their tentacles to form a very basic fishing net for feeding.

All zooids are formed by budding in budding (or growth) zones present in various parts of the siphonophore individual or "colony." Buds mature into particular zooids, for example, those in a cormidium, as they move down the stem toward the posterior end and the stem simultaneously lengthens. The budding zone is often contracted and difficult to identify in preserved material, but in Fig. 2b of a typical physonect, the siphosomal budding zone is clearly visible.

Molecular Phylogeny and Valid Taxa

The new molecular phylogeny of Siphonophora by Dunn et al. (2005b) is based on the nuclear small subunit ribosomal RNA gene 18S and the mitochondrial large subunit ribosomal RNA gene 16S. It shows that Cystonecta (as Cystonectae), without nectophores, is sister to all other siphonophores, termed the Codonophora, or bell-bearers, with nectophores (Fig. 3). The phylogeny also shows the Physonectae to be paraphyletic, whereas the Calycophorae are a monophyletic clade within the Codonophora.

Sexual state has been found to be an important character in determining relationships within the Codonophora. Cystonects are all dioecious (separate sexes), and five discrete physonect families and one unascribed genus within the Codonophora also display dioecy (Table 1). The remaining four discrete physonect families, three further unascribed physonect genera, and all calycophoran families are monoecious, with both sexes developing on the same individual, albeit at different times, to prevent self-fertilization. Monoecy enables cross fertilization between individuals in the deep sea where populations can be very small and mating opportunities limited.

In codonophorans, cormidia first arise as "pro-buds" on a swelling at the anterior end of the siphosome known as the "horn" or siphosomal growth zone (Fig. 2b). In cystonects there is no horn, and zooids arise as independent buds directly on the stem (Dunn and Wagner 2006). This synapomorphy makes the cormidia into integrated units in Codonophora and may explain the huge radiation and diversity within this group, in contrast to the Cystonecta.

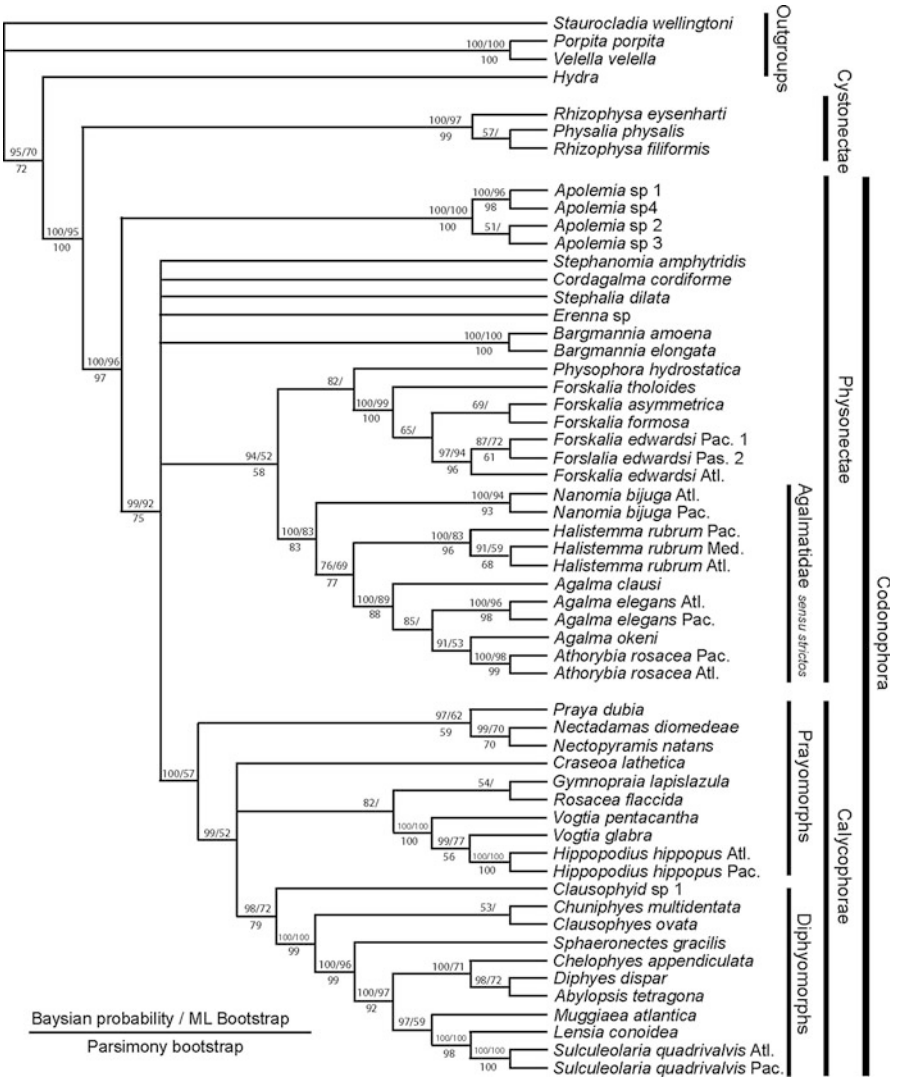

Fig. 3 Molecular phylogeny of Siphonophora from Dunn et al. (2005b, Fig. 6). Consensus tree of all trees for the Bayesian analysis of the combined data set (from an initial 20 million trees). The *left score* above the branch is the Bayesian posterior probability (%), the *right score* above the branch is the ML bootstrap support value (%), and the score below the branch is the MP bootstrap support value (%). The bars to the *right* of the species names indicate clades and grade taxa. Abbreviations: *Atl* Atlantic, *Med* Mediterranean, *Pac* Pacific. For full details of analyses and consensus tree computations, refer to Dunn et al. (2005b) (© Mapstone 2014, Fig. 9)

One hundred seventy-seven species of siphonophore are considered valid at the time of writing (WoRMS Siphonophora List), and these are shown in relation to families, higher ranks, and sexual state in Fig. 4.

Table 1 Siphonophora systematics: higher ranks, valid families, and genera (Extracted from © Mapstone 2014, Table 3, with Stephanomiidae added and a question mark added to *Rudjakovia*; see Table 2)

High rank	Family and subfamily	Genera
I – Cystonecta	01. Physaliidae	*Physalia*
	02. Rhizophysidae	*Bathyphysa*, *Rhizophysa*
II – Codonophora		
Physonectae		
Dioecious families	03. Apolemiidae	*Apolemia*
	04. Erennidae	*Erenna*, *Pararenna*
	05. Pyrostephidae	*Bargmannia*, *Pyrostephos*
	06. Rhodaliidae	*Angelopsis*, *Aranciala, Dromalia*, *Archangelopsis*, *Steleophysema*, *Stephalia*, *Thermopalia*, *Tridensa*
	07. Stephanomiidae	*Stephanomia*
	08. Unascribed *dioecious genus*	*Marrus*
Monoecious families	09. Forskaliidae	*Forskalia*
	10. Physophoridae	*Physophora*
	11. Resomiidae	*Resomia*
	12. Agalmatidae *sensu stricto*	*Agalma, Athorybia*, *Melophysa*
	13. Unascribed monoecious genera	*Cordagalma*, *Frillagalma, Lychnagalma*, and maybe *Rudjakovia*
Calycophorae		
Prayomorphs	14. Prayidae	
	S-f Amphyicaryoninae	*Amphicaryon*, *Maresearsia*
	S-f Prayinae	*Craseoa*, *Desmophyes*, *Rosacea*, *Gymnopraia*, *Lilyopsis*, *Mistoprayina*, *Praya*, *Prayola*, *Stephanophyes*
	S-f Nectopyramidinae	*Nectadamas, Nectopyramis*
	15. Hippopodiidae	*Hippopodius, Vogtia*
Diphyomorphs	16. Clausophyidae	*Chuniphyes*, *Clausophyes*, *Crystallophyes*, *Kephyes*, *Heteropyramis*
	17. Sphaeronectidae	*Sphaeronectes*
	18. Diphyidae	
	S-f Sulculeolariinae	*Sulculeolaria*
	S-f Diphyinae	*Chelophyes*, *Dimophyes*, *Diphyes*, *Eudoxoides*, *Lensia*, *Muggiaea*
	S-f Giliinae	*Gilia*
	19. Abylidae	
	S-f Abylinae	*Abyla*, *Ceratocymba*
	S-f Abylopsinae	*Abylopsis*, *Bassia*, *Enneagonum*

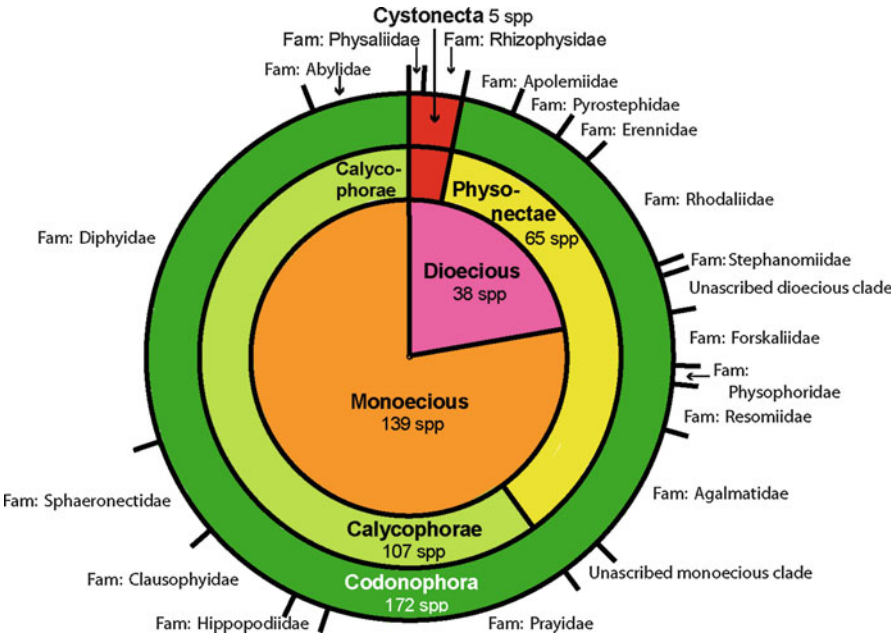

Fig. 4 Siphonophora species. The 177 valid Siphonophora species subdivided into ranks based on Table 1 (Derived from © Gillian Mapstone 2014, Fig. 2C)

Proposed Morphological Phylogeny of the Siphonophora

A tentative phylogeny of the Siphonophora derived from the molecular phylogeny in Fig. 3 is given in Fig. 5. It was proposed by Pugh (2006a) and is based on sexual state and several other morphological characters not previously considered important.

This phylogeny shows that particular diagnostic characters, including those discussed above, might have been important during siphonophore evolution. Other significant characters could have been the position of origin of the zooids on the siphonophore stem, the type of canals on the proximal surface of the nectophore, and the amount of musculature in the nectophore nectosac. Studies on budding (growth) zones and cormidial development in seven siphonophore taxa, together with earlier studies on three other taxa, led Dunn and Wagner (2006) to suggest several key transitions that could have occurred during siphonophore evolution. These include the appearance of a siphosome and pneumatophore in the ancestral siphonophore, the origin of the nectosome in the ancestral codonophoran, and a change from dioecy to monoecy during the evolution of the four monoecious physonect families (and three unascribed genera) and all calycophoran families (Fig. 5, and see Dunn and Wagner 2006, Fig. 7).

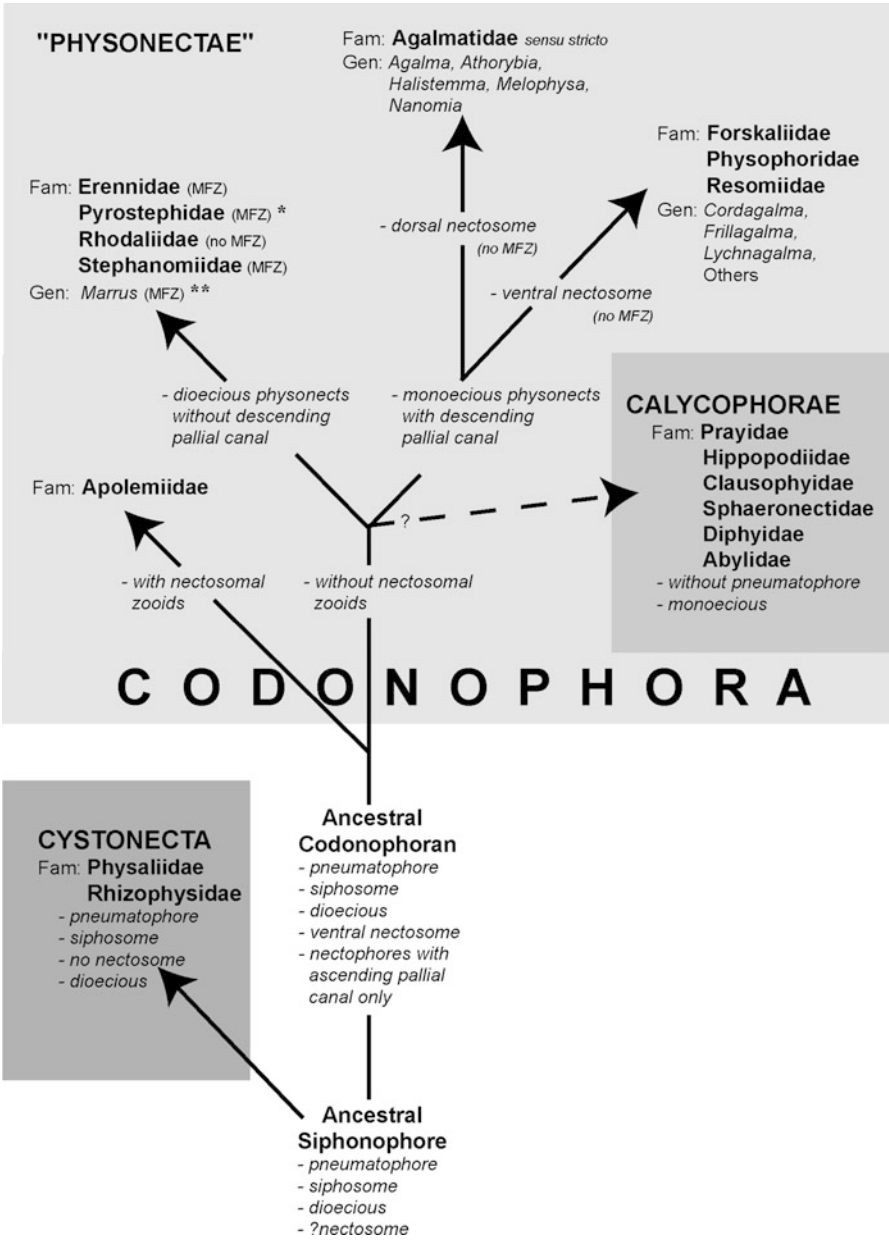

Fig. 5 Possible phylogeny of the Siphonophora (Derived from Pugh 2006a, Fig. 21; Siebert et al. 2013; Pugh and Baxter 2014; © Mapstone 2014, Fig. 10); MFZ – muscle-free zone on nectophore; * – dorsal nectosome and some undescribed species monoecious; ** – one species monoecious

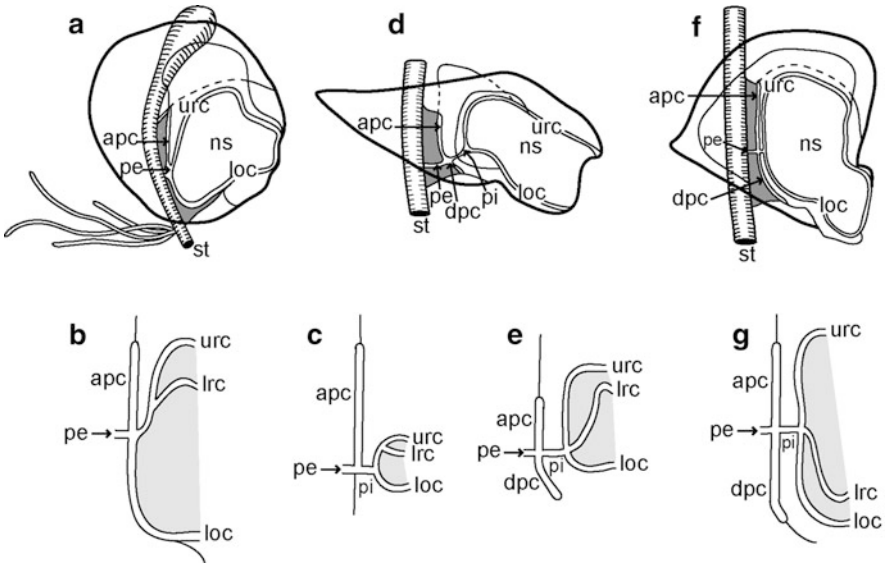

Fig. 6 Pedicular and radial canal arrangements in nectophores of four physonect species. All diagrams through nectophore midline. (**a**, **b**) Dioecious *Apolemia uvaria*; (**c**) dioecious *Bargmannia* sp.; (**d**, **e**) monoecious *Agalma elegans*; (**f**, **g**) monoecious *Nanomia bijuga*; *apc* ascending pallial canal (surface diverticulum), *dpc* descending pallial canal (surface diverticulum), *loc* lower radial canal, *lrc* lateral radial canal, *ns* nectosac, *pe* external pedicular canal, *pi* internal pedicular canal, *st* stem, *urc* upper radial canal; nectophoral muscular lamella shown in *dark gray* in (**a**, **d**, **f**); nectosac shown in *pale gray* in (**b**, **c**, **e**, **g**) (Derived from © Gillian Mapstone 2009, Figs. 5 and 6)

Dunn and Wagner (2006) also suggest that the nectosome of the ancestral codonophoran might first have appeared as a tandem duplication of the siphosome. Siphosomal zooids are taken, by convention, to arise from the ventral side of the stem (Haddock et al. 2005a), and nectosomal nectophores are also thought to have arisen from this same meridian in the first codonophorans (Fig. 5). Later during evolution, the nectosome appears to have twisted relative to the siphosome with the result that, in the family Agalmatidae *sensu stricto*, nectophores arise from the dorsal side of the stem (Fig. 5; Dunn and Wagner 2006).

Another character of importance, which appears to have been modified during codonophoran evolution, is the type of canals present on the proximal surface of the nectophore. These canals, known either as the pallial canals (Mapstone 2014), the mantle canals (Pugh and Baxter 2014), or the ascending and descending diverticula (Mapstone 2009), are important for shedding, or self-amputation, of nectophores and other zooids during autotomy (Mapstone 2009). Thus, they form what is sometimes termed an "autotomy joint." Nectophores senesce (age) as they pass down the nectosome to the posterior end where they split off from the muscular lamella along the line of this canal (Fig. 6) one by one, as more nectophores are added in

the nectophoral budding zone at the anterior end of the nectosome (Mapstone 2009). Nectophores may also be shed for defense, if startled by a predator, leaving loose zooids in the water to create confusion and enabling the siphonophore to retreat (Mackie et al. 1987). Only an ascending pallial canal is present in the dioecious Apolemiidae, Erennidae, Pyrostephidae, Rhodaliidae, and Stephanomiidae, whereas in monoecious physonects a second descending pallial canal is also developed (Figs. 5 and 6).

Other morphological characters of importance during siphonophore evolution are summarized in tabular form and discussed under family headings below (see Table 2).

Cystonecta

A monophyletic clade that is sister to all other siphonophores, as noted above. Cystonects are dioecious, without a nectosome or any bracts in the siphosomal cormidia. The two families referable to this clade are Physaliidae and Rhizophysidae, and typical cystonect morphology is shown in Fig. 2a (long-stemmed cystonect) and Fig. 7 (Cystonect morphology).

The well-known and enigmatic Portuguese man-of-war cystonect, *Physalia*, is large, is pleustonic (lives at the surface) when mature, and has a much enlarged crested float (pneumatophore) propelled by the wind, but no stem (Fig. 7a). It is the only siphonophore with toxins sufficiently powerful to harm humans, although the envenomations reported worldwide in warmer waters rarely lead to death (Fenner 2000). Cormidia arise directly on the underside of the float and the long tentacles stream out to windward (Fig. 7b). Cormidia bud one from another in a series, each termed a "cormidial complex" (Fig. 7c). Indeed, *Physalia* displays the most prolific budding of any siphonophore. A mature specimen has 12 cormidial complexes arising in two groups (Fig. 7b), with, for example, one simpler complex in the oral region, giving a total of c. 13 cormidia. Most cormidia are tripartite, with a gastrozooid and gonodendron and a basigaster separated from the column of the gastrozooid to form a separate ampulla with tentacle (Fig. 7d); in the gastrozooids of all other siphonophores, the basigaster forms a thickened ring around the proximal end of this zooid itself (where nematocyst formation occurs). Most tentacles are convoluted (Fig. 7e) and supported by an extensible membrane, which allows them to contract up near to the float when not feeding. Nematocysts cover the tentacles and are particularly concentrated in the convoluted tentacles (Fig. 7f); however, these concentrations do not constitute true nematocyst batteries, which, in siphonophores, occur on the tentilla (side branches) of the tentacles of all codonophorans except the Apolemiidae (Mapstone 2009, p. 74). In *Rhizophysa*, nematocysts are variably distributed, forming a simple line along each tentacle side branch in *R. eysenhardti* (Fig. 2a), and concentrated into small button-like clusters on the trifid tips of each tentacle side branch in *R. filiformis* (Fig. 7h, i) (Totton 1965).

Table 2 Characters for cystonect and "physonect" families (Derived from © Mapstone 2014, Table 4, with Stephanomiidae added; see this paper also for additional references omitted below; see Fig. 5 for details of fundamental siphonophore characters mentioned below)

	Family	Comments
01.	Physaliidae	Monotypic for *Physalia physalis* (*P. utriculus* considered a junior synonym, Bardi and Marques 2007)
02.	Rhizophysidae	Long-stemmed family of four valid species; *Bathyphysa japonica* a junior synonym of *B. conifera*; SEM (scanning electron microscope) studies of budding sequences described for *B. sibogae*, *Rhizophysa filiformis*, and *R. eysenhardti* (Dunn and Wagner 2006)
03.	Apolemiidae	Long-stemmed family; monophyletic and sister to all other Codonophora, with unique nectophoral palpons on the nectosome. Nectophores distinctive and ridgeless, cormidia dispersed or discrete (pedunculate); gastrozooids with simple tentacles (no tentilla) resembling palpacles of palpons. Monogeneric for *Apolemia*. Two new species include *A. lanosa* and *A. rubriversa* (Siebert et al. 2013) and three older species *A. contorta*, *A. uvaria*, and *A. vitiazi* (*Tottonia contorta* sensu Mapstone 2003 now referable to *A. lanosa*). A number of other species are known to exist (Dunn et al. 2005b; Mapstone 2003, 2009; Siebert et al. 2013) and await full description
04.	Erennidae	Long-stemmed family erected for four species with large prominent straight tentilla, no involucrum, and a rigid terminal process lacking nematocysts. Two genera: *Erenna* (three species) and *Pararenna* (one species). *E. richardi* Bedot, 1904, and a new species *E. laciniata* have large flattened nectophores and large tentilla held close to the body and vibrated to attract prey; two further new species *E. cornuta* and *Pararenna emilyae* have different and also unique tentilla and gastrozooids (Pugh 2001)
05.	Pyrostephidae	Long-stemmed family of five species in two genera: *Bargmannia* (four species), *Pyrostephos* (one species). Pugh (1999a) reviewed the family, introducing two new species (*B. amoena, B. gigas*) and revising two others (*B. elongata*, *B. lata*); *Mica micula* shown to be putative post-larva of a pyrostephid (Grossmann et al. 2013). Nectophores with unique lower-lateral wings and much enlarged triangular thrust block; in *B. elongata* two growth zones on stem and composition of the cormidia studied using SEM (Dunn 2005); pyrostephid cormidia either have oleocysts (modified tentacle-less palpons) (in *Pyrostephos*) or none (in *Bargmannia*) (Pugh 1999a)
06.	Rhodaliidae	Short-stemmed family of eight genera, with four new species including *Archangelopsis jagoa*, *Arancialia captonia*, and two in the genus *Tridensa*, including *T. sulawensis* and *T. rotunda*. Genus *Sagamalia* reduced to junior synonym of *Steleophysema* (WoRMS Siphonophora List). First in situ feeding observations on four species (Hissmann 2005). *Dromalia alexandri* redescribed (Mapstone and Ljubenkov 2013)
07.	Stephanomiidae	Disbanded family reintroduced for single large long-stemmed dioecious species *Stephanomia amphytridis* (Pugh and Baxter 2014); nectosome ventral, nectosac of mature nectophores with muscle-free zone, and other characters (Fig. 5)

(*continued*)

Table 2 (continued)

	Family	Comments
08.	Unascribed *dioecious genus*	Long-stemmed genus *Marrus* Totton, 1954, with muscle-free zone on nectosac and other characters (Fig. 5); new species *M. claudanielis* introduced (Dunn et al. 2005a)
09.	Forskaliidae	Long-stemmed and delicate monotypic family, probably sister to the Physophoridae (Dunn et al. 2005b). Recently revised (Pugh 2003) with two new species added (*Forskalia asymmetrica*, *F. saccula*) and one reduced to a *species inquirenda* (WoRMS Siphonophora List)
10.	Physophoridae	Family with long nectosome but short corm-like siphosome; previously monotypic for *Physophora hydrostatica* with bract present only in larva; second species *P. gilmeri* added by Pugh (2005). Smaller, less colorful, and with bracts retained on adult colony. Tentilla of this family unique
11.	Resomiidae	Long-stemmed family newly introduced for two species previously referred to the Agalmatidae (*Moseria convoluta*, *M. similis*) and now transferred to a new monotypic genus *Resomia* (Pugh 2006a); two tentilla types uniquely present on each tentacle. Three new species *R. dunni*, *R. ornicephala*, and *R. persica* added by Pugh and Haddock (2010)
12.	Agalmatidae *sensu stricto*	Mostly long-stemmed and recently restricted to genera with dorsal nectosome (Fig. 5) and involucrate tricornuate or unicornuate tentilla with typically tightly coiled cnidoband which now includes two short-stemmed genera (*Athorybia*, *Melophysa*) (Dunn et al. 2005b), with two new species of *Halistemma*, *H. transliratum*, and *H. maculatum* introduced and four other *Halistemma* species redescribed (see Pugh and Baxter 2014 for details)
13.	Unascribed monoecious genera	Long-stemmed monotypic genera *Cordagalma*, *Frillagalma*, and *Lychnagalma* now removed from the Agalmatidae for their ventral nectosomes (Fig. 5); new species *C. tottoni* described; *Rudjakovia plicata* considered a valid species, with a dorsal nectosome but sex unknown; it may be transferred to Agalmatidae when more characters are elucidated (Pugh 2006a; Mapstone 2015)

Apolemiidae

The main characters of this family are summarized in Table 2, and the nectosomal palpons are shown in Fig. 8f. Apolemiids are unusual in growing to lengths of 30 m or more (Siebert et al. 2013), longer than any other known siphonophore (Fig. 8a, b). All zooids arise from the ventral meridian of the stem (Fig. 8c), giving apolemiids a ventral nectosome (Figs. 5 and 8c). The nectosome and cormidia are more complex than in other codonophoran families, with at least two different patterns of cormidial organization apparent in the three different *Apolemia* taxa so far investigated (Siebert et al. 2013). Dispersed cormidia occur in *A. lanosa* (Fig. 8a, b) where zooids spread out along the stem (Fig. 8d, g) as soon as the pro-bud leaves the siphosomal horn. In *A. rubriversa* (Fig. 8f) and *A. uvaria* (Fig. 8e), cormidia are pedunculate, with all zooids of one cormidium arising from the peduncle (or pedicel) of the first

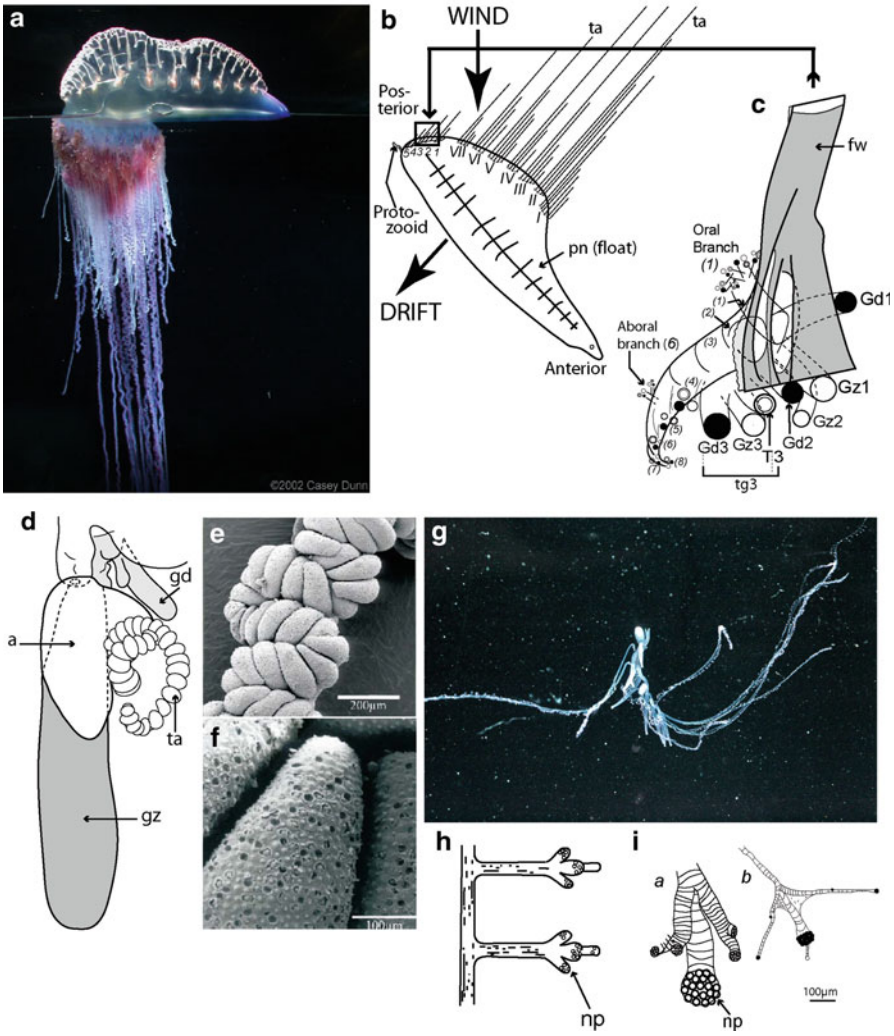

Fig. 7 **Cystonect morphology.** (**a**) Atypical *Physalia physalis*, pleustonic (lives at surface), with much enlarged pneumatophore, no stem, cormidia arising directly from underside of pneumatophore; (**b**) left-handed drifting specimen viewed from above – added numbers 1–5 identify oral cormidial groups, while numbers I–VII identify main cormidial groups – note how *Physalia*'s surface float drifts to starboard with the wind on a broad reach; (**c**) oral cormidial complex number 2 viewed from inside the float – note groups 3–8 are tripartite, with more tripartite groups on oral and aboral side branches – with numbers in brackets added to identify tripartite groups; (**d**) a developing tripartite group from main cormidial complex number VI; (**e**) SEM of part of contracted *Physalia* tentacle; (**f**) detail from (**e**), with darker holes marking discharged nematocysts; (**g**) *Rhizophysa filiformis* (see Fig. 2 legend for long-stemmed cystonect features); (**h**, **i**) *R. filiformis* trifid nematocyst pads; Labels: *a* ampulla (basigaster), *fw* float wall, *gd* gonodendron, *gz* gastrozooid, *np* nematocyst pad, *pn* pneumatophore (float), *t* tentacle, *ta* tentacle with ampulla

gastrozooid to be formed, on the siphosomal horn (Siebert et al. 2013). These fundamental features of zooid budding are concluded by Siebert et al. (2013) to be homoplastic in codonophorans.

In young specimens of *Apolemia uvaria*, pedunculate cormidia are clearly separated from others by bare lengths of stem (Fig. 8e). As growth proceeds, the naked stem portions become partially or completely obscured by prodigious budding of the pedunculate cormidia, as well shown by Siebert et al. (2013, Fig. 18c). In *A. lanosa* the siphosome becomes very long and extends a prominent curtain of "tentacles" for feeding (Fig. 8g). This curtain comprises mainly palpacles from the numerous palpon clusters on the stem, and also many fewer thin tentacles from the gastrozooids (Siebert et al. 2013). Each simple palpacle or tentacle bears a narrow band of nematocysts down one side for catching prey (Mapstone 2003, Fig. 12e, f) but no true tentilla. Stem length is further increased in *A. lanosa* during growth by interpolation of secondary gastrozooids and more palpon clusters, as shown in Fig. 8d.

Erennidae

In this, and all remaining codonophorans, taxa are characterized by the presence of tentilla, or complex stinging batteries, on the side branches of their tentacles. For physonects, these tentilla are diagnostic, but calycophoran tentilla are rather uniform and of little or no diagnostic value. Each tentillum includes a pedicel, thickened cnidoband, and (usually) a thin extensible terminal filament. Each cnidoband is packed with rows of nematocysts, with more on the terminal filament(s) of almost all species. It is the nematocysts, or cnidae, which deliver toxins to the prey, either by penetration or entanglement, and more nematocyst types are found in siphonophores than in any other hydrozoans. Different types of nematocysts, their distribution across siphonophore families, and suitability for different types of prey are summarized by Mapstone (2014, Table 6).

Erennids have unique and exceptionally large tentilla, with a straight cnidoband of small haploneme nematocysts, but no larger heteroneme nematocysts (typical of other codonophorans). Also, uniquely, the thick terminal filament completely lacks nematocysts and instead, in one species at least, bears a pair of distal red lures to attract prey (Table 2, Fig. 9b). Species are dioecious, with nectophores having only an ascending pallial canal on the proximal surface (see Fig. 6c) and a muscle-free zone (MFZ) at the proximal end of the nectosac (Fig. 5). The nectosome is typically long (Fig. 9a) to very long, without any nectosomal polyps, and the nectophores

Fig. 7 (continued) (basigaster), *tg* tripartite group (**a** © Casey Dunn Brown; **b**–**d**, **i** © Gillian Mapstone 2014, Fig. 2 insets Aa, Ab, Fig. 7; for details of published figures and references from which drawings were derived, see relevant figure legends; **e**, **f** from Bardi and Marques 2007, with permission from Iheringia Série Zoologia; **g** © Larry Madin WHOI; **h**: Kawamura 1910, Fig. 5d)

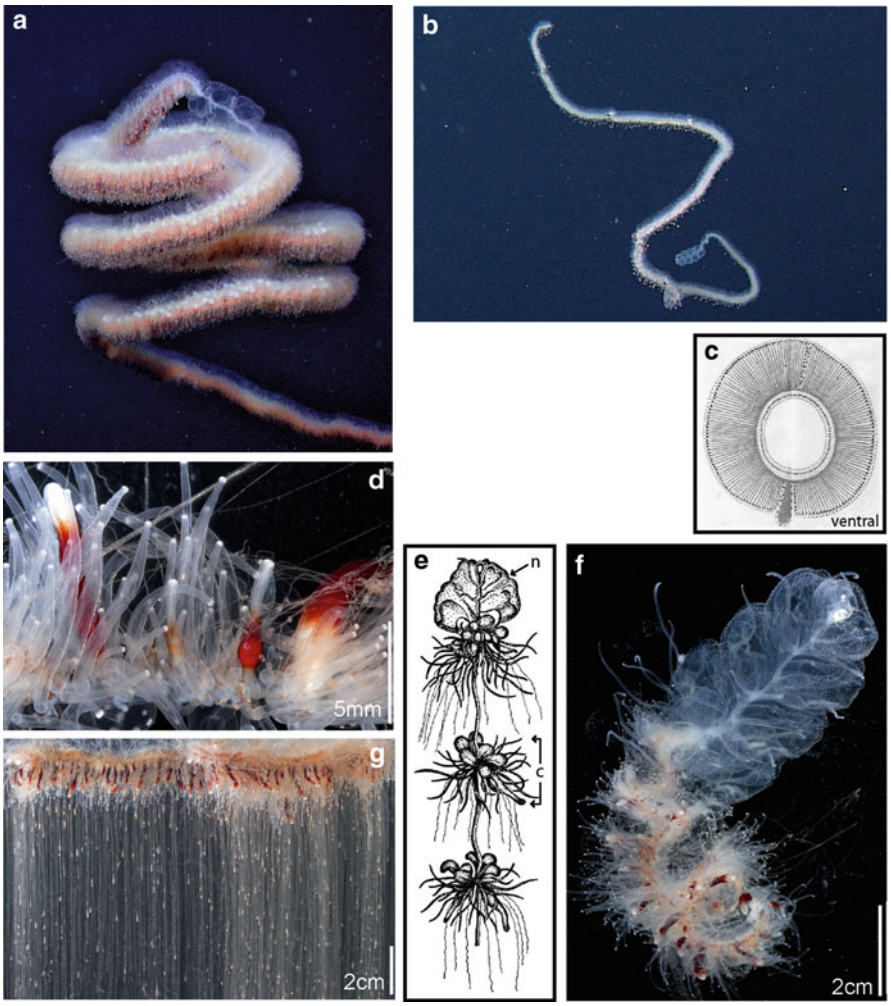

Fig. 8 Apolemiid morphology. (**a**, **b**, **d**, **g**) *Apolemia lanosa* from Monterey Bay, (**a**) whole colony, from 1,150 m (© 2005 MBARI); (**b**) whole colony, from 670 m (© 2011 MBARI); (**c**) *A. uvaria* TS through stem showing ventral zooid meridian (Korotneff 1883, pl. 14 Fig. 9); (**d**) *A. lanosa* detail of cormidium with two large gastrozooids (large red zooids), many longer palpons, and a secondary gastrozooid (small red zooid) (© Stefan Siebert); (**e**) *A. uvaria* young colony, showing pedunculate cormidia (Gegenbaur 1853, pl. 18 Fig. 1); (**f**) *A. lanosa* nectosome with siphosome fragment; note nectophoral palpons emerging from between the nectophores (© Stefan Siebert); (**g**) *A. lanosa* part of siphosome with extended "tentacle" curtain (no tentilla); note many red gastrozooids (© Stefan Siebert)

typically large and flattened, often with black pigment on the radial canals and other zooids (Pugh 2001). Erennids live at great depths, in the twilight zone where prey is scarce. In the species with red lures, and probably also the other *Erenna* species (Pugh 2001), the siphosome is permanently contracted, and the tentilla always held

close to the body. The lures emit red light (from bioluminescence) and together resemble a shoal of small deep-sea fish in the water. These attract deep-sea bristlemouth fish, which are of similar size and color. These fish are thought to swim into the lures, to be immediately stunned by nematocysts on the long straight cnidobands and then ingested by the gastrozooids, together with some lures (Haddock et al. 2005b).

Pyrostephidae

Most family characters are summarized in Fig. 5 and Table 2. Additionally, pyrostephids lack palpacles (palpon tentacles), and each cormidium contains only either a single unique siphosomal tentacle (or tentaculozooid) (*Bargmannia*) or a modified palpacle-less palpon termed an oleocyst (*Pyrostephos*) (Dunn 2005; Pugh 1999a). This latter zooid contains an oil-filled vesicle which gives extra support to the heavy, vermillion red stem (Totton 1965). Tentilla are also unique in pyrostephids, with a cnidoband of mostly very small nematocysts (desmonemes and acrophores) and only a few larger stenoteles at the proximal end (Fig. 9c). An axial gastrovascular canal penetrates the length of the terminal filament and is probably used to extend the cnidoband during prey capture, since pyrostephid tentilla lack the paired elastic strands found in the tentilla of most other siphonophores (Totton 1965). Pyrostephid terminal filaments bear many small nematocysts similar to those present in the cnidoband (Mapstone 2014, Table 7).

The cormidial composition of *Bargmannia elongata* was revealed during an elegant SEM study by Dunn, who also identified two growth zones: a siphosomal horn on which pro-buds develop, with subdivision of each pro-bud into one cormidium, and a nectophoral growth zone where individual nectophores develop (Dunn 2005, Table 2). Each cormidium was found to be completely regular and also directionally asymmetric.

The distribution of *Bargmannia* species is variable and for some species difficult to assess due to problems of past misidentification (Pugh 1999a). Both *B. elongata* and *B. amoena* occur in the Atlantic, as does *B. lata* (Pugh 1999a), while in the Pacific only *B. elongata* and *B. lata* have so far been positively identified (Mapstone 2009). *Pyrostephos vanhoeffeni*, in contrast, is restricted to the southern hemisphere (Mapstone 2014, Table 1) with an extensive distribution map published recently by Lindsay et al. (2014). These authors found young specimens of *P. vanhoeffeni* concentrated close to the Antarctic coast (nectophores previously misidentified as *B. elongata*), while larger and more mature nectophores were found further north, in the open ocean. Indeed, there are records from as far north as 35°S in the Atlantic and Pacific (Lindsay et al. 2014, map 3). Despite this, the name *P. vanhoeffeni*, meaning "spiral of fire," was originally applied to some big specimens collected not too far from the coast, near to the ice edge at 90°E. These were brightly colored, up to several meters long, and first described by Moser (1925), with more color notes being given by Totton (1965, p. 78), who pointed out that the stem, gastrozooids, and tentilla are all bright red, while the nectophores are pale pink with bright red ostia.

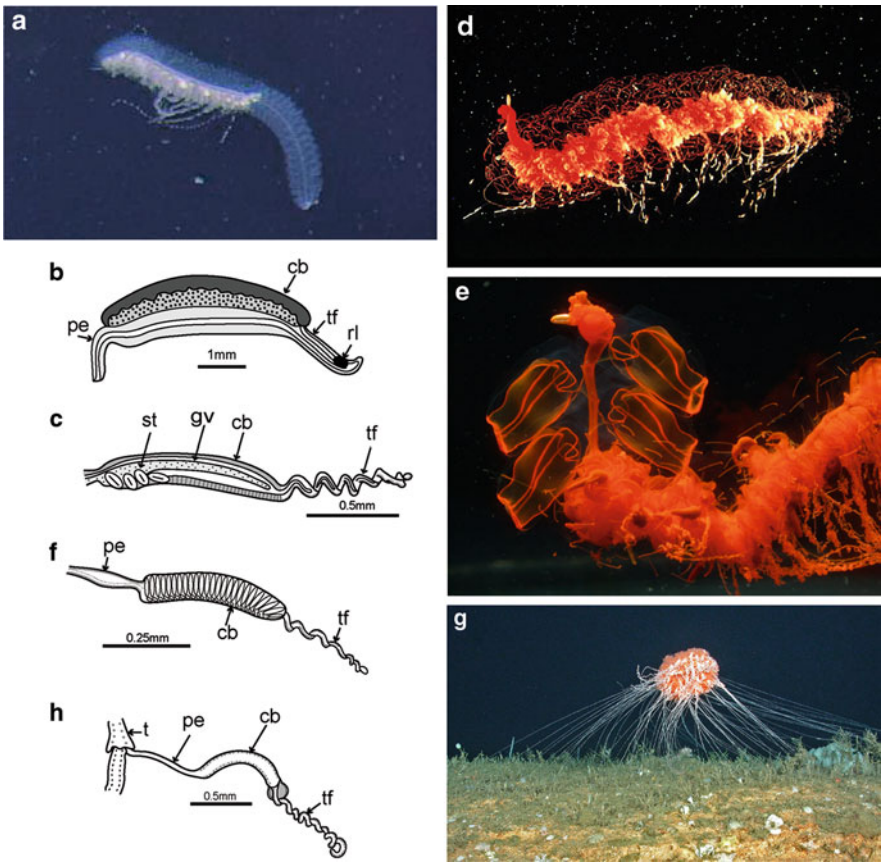

Fig. 9 Morphology of other dioecious physonects. (**a**) *Erenna laciniata* (© JAMSTEC, https://fbox.jamstec.go.jp/public/XMH0AAdKtcvAt7cBnXVJOhXGd3gQe6hkGsJUttHV-l Si; the organism in this image was identified by Dr. Dhugal Lindsay based on examination of the collected specimen); (**b**) *Erenna richardi* tentillum; (**c**) *Bargmannia elongata* tentillum; (**d**) *Marrus claudanielis* colony, nectophores autotomized (© Marsh Youngbluth); (**e**) *Marrus orthocanna*, nectosome, and first part of siphosome (© Casey Dunn); (**f**) *Marrus orthocanna* tentillum; (**g**) *Dromalia alexandri* (permissions@wiley); (**h**) *Tridensa sulawensis* tentillum (**b**, **c**, **f,** and **h** © Gillian Mapstone 2014, from Fig. 12). Labels: *cb* cnidoband, *gv* gastrovascular canal, *pe* pedicel, *rl* red lure, *st* stenotele nematocysts, *t* tentacle, *tf* terminal filament

Rhodaliidae

This is one of the most species-rich "physonect" families, comprising 14 species in eight genera (Fig. 4; Table 2; WoRMS Siphonophora List). Rhodaliids have undergone more speciation than long-stemmed and pelagic physonects due to their semi-benthic habit, which has led to greater geographical isolation and more restricted ranges for all species (see Mapstone 2014, Table 1, for summaries of two rhodaliid distributions and abundances: *Dromalia alexandri* and *Rhodalia miranda*). The

larvae and life cycles of rhodaliids are unknown, and main diagnostic characters differ from those of pelagic physonects in that nectophores are simple and lack any surface ridges and have a nectosac that is muscular throughout and a siphosome which is coiled up into a near-spherical corm with cormidia crowded around it in whorls and growing outward rather than being spread along a long linear stem.

Important diagnostic characters for rhodaliids include shape and surface texture of the enlarged pneumatophore and its associated gas gland or aurophore, type of corm developed (thin walled, thick walled, or solid throughout), type of siphosomal cormidia present (on separate stems or several on a common stem), and type of bracts developed. In *Dromalia alexandri*, from off Southern California (Fig. 9g), the pneumatophore is turreted, the aurophore is papillate, and several siphosomal cormidia develop on common stems called cormidial units, with up to three cormidia per stem. Furthermore, growth of cormidia continues throughout life, with units circling around the corm up to eight times in total. *D. alexandri* has a unique body form, has been recently redescribed by Mapstone and Ljubenkov (2013), and its characters compared to those of six other similar genera (Mapstone and Ljubenkov 2013, Table 3).

Siphosomal cormidia of rhodaliids are either monogastric (each arising separately on an individual stem), or polygastric, with several cormidia arising from a common stem and somewhat resembling a tree. These structures are termed "cormidial units," and mature units from the first and second whorls of *Dromalia alexandri* are illustrated by Mapstone and Ljubenkov (2013, Figs. 7 and 8). In other rhodaliids, cormidia production ceases once two whorls are formed. An important character of rhodaliids is the presence of two types of gastrozooids instead of one: type II gastrozooids each have a long tentacle bearing many tentilla for prey capture, while type I gastrozooids have either only a small tentacle or none, and lack any tentilla. When a rhodaliid attaches to the substrate, it deploys many type II tentacles in a three-dimensional array (Fig. 9g), and prey blunders into this net to be stunned and held by the tentilla. Type I gastrozooids then extend out to these tentilla, "hoover up" the prey, and digest it. Feeding has been described in three rhodaliid species by Hissmann (2005), who identified prey items from small copepods and amphipods to larger amphipods and fish larvae, which are captured by the type II gastrozooids and digested by the type I gastrozooids.

Tentilla of rhodaliids are of typical physonect structure, with a pedicel, elongate cnidoband (sometimes with a bilobed distal end; see Fig. 9h) of mostly larger and sometimes also smaller nematocysts, and an elongate terminal filament of many small nematocysts, of one or two types (where known) (Mapstone 2014, Table 7). Bracts occur in the cormidia, typically arise from elongate bracteal lamellae, and are species-specific for those species in which cormidia have been collected and studied (Mapstone and Ljubenkov 2013).

Stephanomiidae

This family has only recently been reinstated by Pugh and Baxter (2014) for a single large species *Stephanomia amphytridis* (Table 2) first figured from the siphosome only

in 1807 by Lesueur and Petit, and subsequently found with nectosomal zooids at a number of temperate and tropical locations worldwide (except the South Pacific). The name Stephanomiidae was introduced by Huxley for a second siphosome he found off the east coast of Australia in 1859, which was found again by Bigelow in the tropical east Pacific in 1911, and again by Mapstone from the Flores Sea in Indonesia in 2004, this time with the nectosome as well. All these latter specimens have since been referred to the agalmatid species *Halistemma foliacea* (see below).

Stephanomia amphytridis is a large and prominent species reaching up to 5 m in length, with 25 or more very large nectophores and a distinctive semirigid orange siphosome which is enclosed by many robust bracts (Pugh and Baxter 2014). The orange color in the siphosome is due to pigmented gastrovascular fluid (Dunn et al. 2005b) and not to orange pigment in the stem and zooids, as in the two *Marrus* species discussed below. *S. amphytridis* is dioecious (with separate sexes), with a ventral nectosome, a muscle-free zone on the nectosacs of mature nectophores, sinuous lateral radial canals, and only an ascending pallial canal on the proximal nectophore surface (see Fig. 5). The pattern of nectophore ridges is similar to that of *Halistemma* species, but the vertical-lateral ridges form a complex that is unique to *S. amphytridis*. The tentilla of the long tentacles also somewhat resemble those of *Halistemma* species, since the cnidoband is loosely coiled and there is only a single terminal filament; but an involucrum is absent and the nematocysts also seem to be different. In the cnidoband nematocysts are all large, with thin ones filling most of the cnidoband, but unidentifiable and fatter ones, which could be stenoteles, flanking it on both sides. Similarly, two types of smaller nematocysts occur on the terminal filament, but these could not be positively identified, although it is certain that they were not the usual acrophores and desmonemes (Pugh and Baxter 2014, and see Mapstone 2014, Table 6, for summary of typical physonect nematocysts).

Amongst the material of *Stephanomia amphytridis* studied by Pugh and Baxter (2014), seven small *Nectalia* postlarvae were identified and described, each about 7–8 cm in length. Their nectophores resembled those of the mature individuals, but the siphosome was very short and had only just started to grow. It comprised a single gastrozooid (the protozooid) with its tentacle, surrounded by some distinct elongate larval bracts. The tentacle bore a number of unique larval tentilla unlike those found in any other physonect, and these contained three types of nematocysts. Species of *Halistemma* also pass through a *Nectalia* stage in their life cycle, but have quite different larval tentilla.

Unascribed Dioecious Genus

The genus *Marrus* was assigned to this group by Pugh (2006a, Fig. 21) for the muscle-free zone on the nectosac (Fig. 5). *Marrus* is an enigmatic genus, with two well-recognized species (*M. claudanielis* and *M. orthocanna*; see Dunn et al. 2005a), one doubtful species (*M. antarcticus*; see Mapstone 2009), and a fourth which requires transfer to a different genus (*M. orthocannoides*; see discussion in Dunn et al. 2005a; Mapstone 2009). Two recently studied species are striking *in vivo*.

M. claudanielis (Fig. 9d) was introduced and described by Dunn et al. (2005a); it has a stiff stem which never relaxes (similar to *Agalma okeni*, see below) and a thick red zooid-covered siphosome that spirals around to the posterior end and is surrounded by a characteristic "halo" of transparent bracts (Fig. 9d). The appearance of *M. orthocanna* is similar (Fig. 9e). *Marrus* species are very fragile and often autotomize their zooids when illuminated or otherwise disturbed. The colony in Fig. 9d has already lost its nectophores, but in the small *M. orthocanna* colony shown in Fig. 9e, the nectophores are still intact. Other images of *M. orthocanna* taken under the Arctic ice (see Mapstone 2009, frontispiece A-B) show a larger individual with at least 12 nectophores, and up to 37 nectophores or more have been recorded in other specimens (Andersen 1981).

Nectophores of *Marrus* species are ridged, with straight red pigmented radial canals and a pair of red-yellow chromatophores on each side of the ostium (possibly for disruptive coloration). The nectosac has a large muscular-free zone, and there is only an ascending pallial canal (surface diverticulum) on the proximal nectophore surface. Nectophore ridges are of diagnostic importance in many physonect species, and in *Marrus* upper-lateral ridges divide distally in *M. orthocanna*, but not in *M. claudanielis* (Dunn et al. 2005a). Circa 30 mature gastrozooids have been found in the largest *Marrus* colonies studied, and the tentilla on their tentacles are either straight (Fig. 9f) or only loosely coiled. Gonodendra are also numerous on the siphosome and include equal numbers of male and female gonophores in *M. orthocanna* (Andersen 1981), which is monoecious, but gonophores of only one sex in *M. claudanielis*, which is dioecious (Dunn et al. 2005a). Bracts are large and kite-shaped in both species, with a prominent orange band of nematocysts and associated ectodermal cells on the upper surface. In *M. orthocanna*, this band is relatively short and straight (Fig. 9e), while in *M. claudanielis* it is longer and arc-shaped (Fig. 9d); this character is particularly useful for separating the two species *in vivo*.

Forskaliidae

This is the first monoecious physonect family listed in Tables 1 and 2 and has a ventral nectosome and a descending pallial canal (descending surface diverticulum) on the proximal nectophore surface (Fig. 5). It is monotypic for *Forskalia* and includes six valid species (WoRMS Siphonophora List). The "pallial canals" are shorter than those shown in Fig. 6d (see Totton 1965, Figs. 57 and 58b) and the internal pedicular canal (pi) much longer (see Pugh 2003, Figs. 2, 9, 17, 25, 34, and 40), because nectophores are particularly flattened along the upper-lower axis and extended along the proximal-distal axis (see Mapstone 2009, Fig. 1b for axes).

Forskaliids are fragile animals and distributed around the globe chiefly in the warmer waters of tropical and warm-temperate latitudes. SCUBA divers have frequently observed them in the Mediterranean and western Atlantic, although forskaliids have also been collected by manned submersibles in the Alboran Sea (western Mediterranean) and the Bahamas (Pugh 2003). Observations on colonies

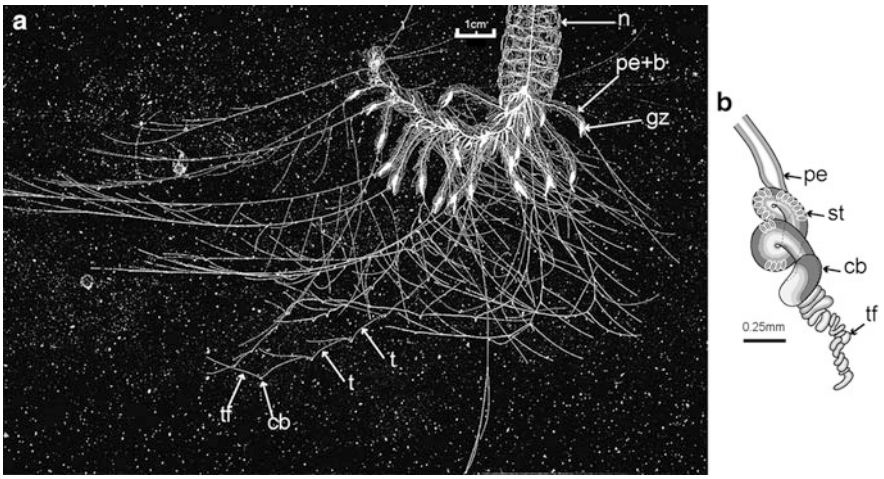

Fig. 10 Morphology of monoecious physonects 1. Forskaliidae. (**a**) *Forskalia* sp. (© Inter-Research, revised from Luo et al. 2014); (**b**) *Forskalia edwardsi* tentillum (© Gillian Mapstone 2014, Fig. 12H). Labels: *cb* cnidoband, *gz* gastrozooid, *n* nectophores, *pe* pedicel of gastrozooid, *pe + b* gastrozooid pedicel with bracts, *st* stenotele nematocysts in the cnidoband, *t* tentacle, *tf* terminal filament

show them to be mostly snake-like and elongate, with the siphosome longer and broader than the nectosome (see WoRMS Siphonophora List image of *Forskalia edwardsi*), although in some *Forskalia* species (Fig. 10a), the siphosome appears to be short (*F. tholoides*, *F. asymmetrica*; Pugh 2003). One species, *F. formosa* has been observed in the Mediterranean swimming powerfully and simultaneously rotating anticlockwise (Pugh 2003). However, in general, *Forskalia* species swim more slowly than most other physonects, because of their long and broad siphosome, and have a small pneumatophore for greater maneuverability (Biggs 1977). The specimen in Fig. 10a is relaxed for feeding, with the nectosome uppermost, although other forskaliids have been observed hanging vertically in the water with the siphosome uppermost (Biggs 1977).

The family review of Pugh (2003) noted that *Forskalia edwardsi* could easily be distinguished from *F. contorta* in the field by the presence of a small distinctive sulfur-yellow spot at the junction of the upper radial canal and the ostial ring canal. Both species have been collected in the Mediterranean and western North Atlantic, and also from the Indian Ocean and Far Eastern seas (Pugh 2003). Another less common species from off India, which is also present in the Gulf of California and the North Atlantic (although not the Mediterranean), is *F. tholoides*, with a distinctive bell-shaped (or fir cone-like) nectosome of nectophores that lack axial wings; this species was introduced in 1888 by Haeckel (WoRMS Siphonophora List). A fourth species is *F. formosa* of which 20 specimens have been collected in the western Mediterranean and two in the Bahamas (Pugh 2003). *F. asymmetrica,* a fifth species, is of similar abundance with 15 specimens known so far from the

western Mediterranean, Bahamas, and canyons off Woods Hole in the NW Atlantic (see WoRMS Siphonophora List); the mean depth for this species is 598 m. The final forskaliid species, *F. saccula*, is known only from one young specimen collected close to the surface in the Sargasso Sea and introduced by Pugh (2003).

In forskaliids, gastrozooids are held away from the siphosomal stem on long pedicels, and the tentacles dangle down outside this cylinder as shown by Pugh (2003, Fig. 16). Bracts occur on both the gastrozooid pedicels and the stem (Fig. 10a, Biggs 1977), and those on the pedicels provide buoyancy for the heavy gastrozooids. Four types of bracts have been found in most species: three types on the gastrozooid pedicels and a fourth on the stem; these are illustrated and described by Pugh (2003) for all six forskaliid species. Also on the stem are gonodendra, comprising gonophores of both sexes (monoecious – sexes maturing at different times), and gonopalpons, which can be species-specific (Pugh 2003).

The tentacles of forskaliids have regularly spaced tentilla, typically 15 per tentacle spaced 5 mm apart (Fig. 10a, Biggs 1977). Each tentillum (Fig. 10b) has a short pedicel, as seen in Fig. 10a, no involucrum, and a loosely coiled orange-red cnidoband composed mainly of homotrichous anisorhizas with some larger lateral stenoteles (Mapstone 2014, Table 7). Beyond the cnidoband, a very long terminal filament extends for feeding (Fig. 10a), and each such filament bears a repeating pattern of small nematocysts including a pair of desmonemes, two pairs of acrophores, a pair of desmonemes, and so on (Mapstone 2014, Table 6). Forskaliids are known to sting fishermen and SCUBA divers badly when contact is made with the tentacles. Copepods are the main prey, together with various other small planktonic organisms (Purcell 1983).

Physophoridae

This family is monoecious, with a ventral nectosome, a siphosome reduced to a corm, and unique encapsulated tentilla which may be jiggled like small copepods to act as lures. It is monogeneric for the genus *Physophora* and includes two species, *P. hydrostatica* and *P. gilmeri. P. hydrostatica* is ubiquitous with a truly cosmopolitan distribution (Mapstone 2014, Table 1), while *P. gilmeri* is rare (Table 2) and known so far only from nine specimens around the Bahamas (Pugh 2005) and one off Japan (Lindsay and Miyake 2009).

The nectosome is typical of long-stemmed physonects and bears up to 12 nectophores arranged in two rows (although all originate from a single ventral meridian). A ring of up to 36 prominent palpons fringes the outer edge of the spherical corm below, each terminating in an ampulla of large microbasic mastigophore nematocysts, which can inflict a painful sting. The ampulla of each palpon is white in *P. hydrostatica* (Fig. 11a) and orange in *P. gilmeri*. Each mature cormidium comprises three palpons, one smaller gastrozooid with tentacle, and a reproductive body on a single stalk which subdivides immediately into a male gonodendron branch and a female gonodendron branch (Fig. 11b*b*). In addition, the cormidia of *P. gilmeri* each contain one or more bracts of two types (Pugh 2005).

In *P. hydrostatica*, a single bract develops only in the larva, for buoyancy until some large palpons develop, when the bract is lost (Totton 1965). In *P. gilmeri* buoyancy provided by the palpons is apparently supplemented in mature colonies by the large bracts; whether a single larval bract is produced in the larva of this species is unknown.

The tentilla of physophorids differ from those of all other codonophorans in being enclosed within a capsule on a long pedicel, and lacking any terminal filament. The cnidoband is up to 5 mm long, of many small anisorhizas flanked by a few larger microbasic mastigophores (Mapstone 2014, Table 7); it inverts during growth and then unwinds into a chaotic spiral and discharges through a pore near the proximal end of the capsule (Fig. 11c).

Resomiidae

This small family, introduced in 2006 and summarized in Table 2, includes five long-stemmed species which are very fragile, have a rigid stem, and are transparent except for buttons or arcs of nematocysts at the tips of the bracts, faintly tinted gastrozooids, and palpons (Pugh and Haddock 2010). They also have remarkable tentilla which transform during growth from a spirally coiled form into a (typically) zigzag form. This has been studied in detail in all species, and the process somewhat resembles cnidoband rearrangement in the capsulate tentilla of species in the physonect genus *Physophora*, although the latter differs in having a shortened swollen siphosome and no terminal filament on the tentilla, as described above. In *Resomia*, after coiling, a transparent involucrum typically grows over the entire cnidoband and extends on to form a distal tube into which the terminal filament is withdrawn when not feeding. Once covered, the cnidoband uncoils and rearranges itself into three zags, with the double elastic band (employed during tentillum activation) connecting only the proximal and distal ends of the cnidoband (see Pugh 2006a, Figs. 11 and 18). One exception to this growth pattern is *R. ornicephala* in which the involucrum grows out to float above the cnidoband instead of enclose it, and is pigmented. The pigment fluoresces under violet and blue excitation making the involucrum resemble a bird's beak, with a central green strip flanked by two pairs of yellow spots (Pugh and Haddock 2010). In the field the long tentacles are repeatedly relaxed and tugged up through the water in a jiggling movement. *R. ornicephala* inhabits a restricted depth range 164–298 m in Monterey Bay and must compete with the more abundant small physonect *Nanomia bijuga* for food. In the dim downwelling light at this depth, the lure of *R. ornicephala* may either fluoresce to attract krill prey or resemble a shoal of krill in outline when all the tentacles are extended (Pugh and Haddock 2010).

Few resomiids have been collected worldwide, with two species found only in the Southern Ocean and the remainder in warmer oceanic waters, mainly the NE Pacific, either off or within Monterey Bay, in the Gulf of California, or in the Tongue of the Ocean region of the Bahamas in the Atlantic. Although a lure has been postulated for attracting prey in *Resomia ornicephala*, the type of prey

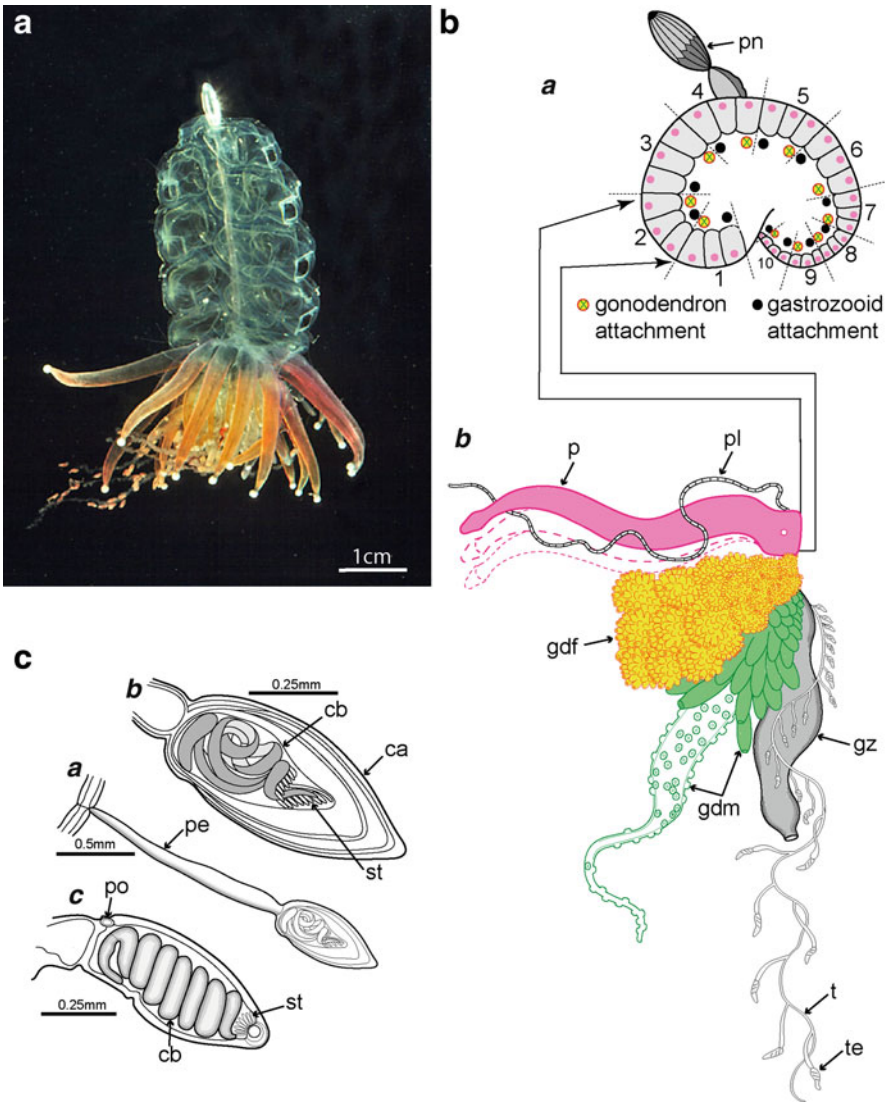

Fig. 11 Morphology of monoecious physonects 2. Physophoridae. (**a**) *Physophora hydrostatica* (© Larry Madin WHOI); (**b*a***) diagram of posterior surface of corm bearing ten cormidia; (**b*b***), one cormidium exploded; (**c**) tentilla, *a* whole tentillum, with long pedicel; *b* mature tentillum capsule with cnidoband reversed, uncoiled chaotically and ready for discharge; *c* less mature capsule with cnidoband already reversed but still spirally coiled (© Gillian Mapstone 2014, Fig. 6Ba, b; Fig. 12Fa–c; see these figure legends for references from which drawings were derived). Labels: *ca* capsule, *cb* cnidoband, *gz* gastrozooid, *gdf* female gonodendron, *gdm* male gonodendron, *p* palpon, *pe* pedicel, *pl* palpacle, *po* pore, *pn* penumatophore, *st* stenotele nematocysts, *t* tentacle, *te* tentillum

captured by other resomiids is unknown. Prey must be immediately stunned by the formidable battery of nematocysts on the long and folded cnidoband (Mapstone 2014, Tables 6 and 7), using the method described for other physonects by Mapstone (2014). So far, feeding in situ has not been observed in any resomiid, and, indeed, in one species, *R. dunni*, only a single well-developed gastrozooid is present on the siphosome.

Agalmatidae Sensu Stricto

This most species-rich pelagic family of all physonects (Fig. 4) was shown to be a distinct monophyletic clade by Dunn et al. (2005b, Fig. 3) and was delimited by Pugh (2006a) to five genera only. Diagnostic features of the family are listed in Table 2, with the most distinctive being the twisting of the nectosome relative to the siphosome resulting in nectophores arising from the dorsal side of the stem and siphosomal cormidia from the ventral side. This is well illustrated in *Halistemma foliacea* by Pugh and Baxter (2014, Fig. 60). The small species *Nanomia bijuga* is the most common physonect worldwide (Fig. 12c) between 55°N and 59°S, with numerous records from all oceans except the North Atlantic (Mapstone 2014, Table 1). The arrangement of zooids in one cormidium is shown in Fig. 12d, and detailed zooid composition of the cormidia is given by Dunn and Wagner (2006). The tentillum comprises a red cnidoband of circa three coils, with an involucrum covering the first coil, and a single distal terminal filament (Fig. 12e).

Nanomia bijuga feeds on a range of small prey, including copepods, decapod larvae, and, in Monterey Bay in particular, various young stages of krill (Pugh and Haddock 2010). As the physonect remains motionless in the water with its tentacles extended for feeding, prey becomes entangled in the long dangling terminal filament; its movements cause discharge of the cnidoband, which unwinds and slaps onto the prey, stunning it instantly, as described in Mapstone (2014, Fig. 15a–c). Recently, the juvenile squid *Chiroteuthis calyx* has been found to mimic *Nanomia bijuga* in Monterey Bay, where it avoids predators by hanging vertically among *N. bijuga*, a species shunned by predators because of its stinging batteries (the tentilla) and low food value (Burford et al. 2014).

Other well-known but less abundant species in the family Agalmatidae *sensu stricto* include *Agalma elegans*, *A. okeni*, and *Halistemma rubrum*. In the past, loose nectophores of *A. elegans* have sometimes been difficult to distinguish from those of *H. rubrum*, but a recent and comprehensive review of the genus *Halistemma* by Pugh and Baxter (2014) has resolved this problem. *Halistemma* nectophores are more truncate than those of *Agalma* species when mature, with much shorter more truncate axial wings separated by a prominent thrust block (see Pugh and Baxter 2014, Fig. 115). *H. rubrum* nectophores display a distinctive pattern of incomplete ridges on the upper surface, whereas all those of *A. elegans* are complete (see

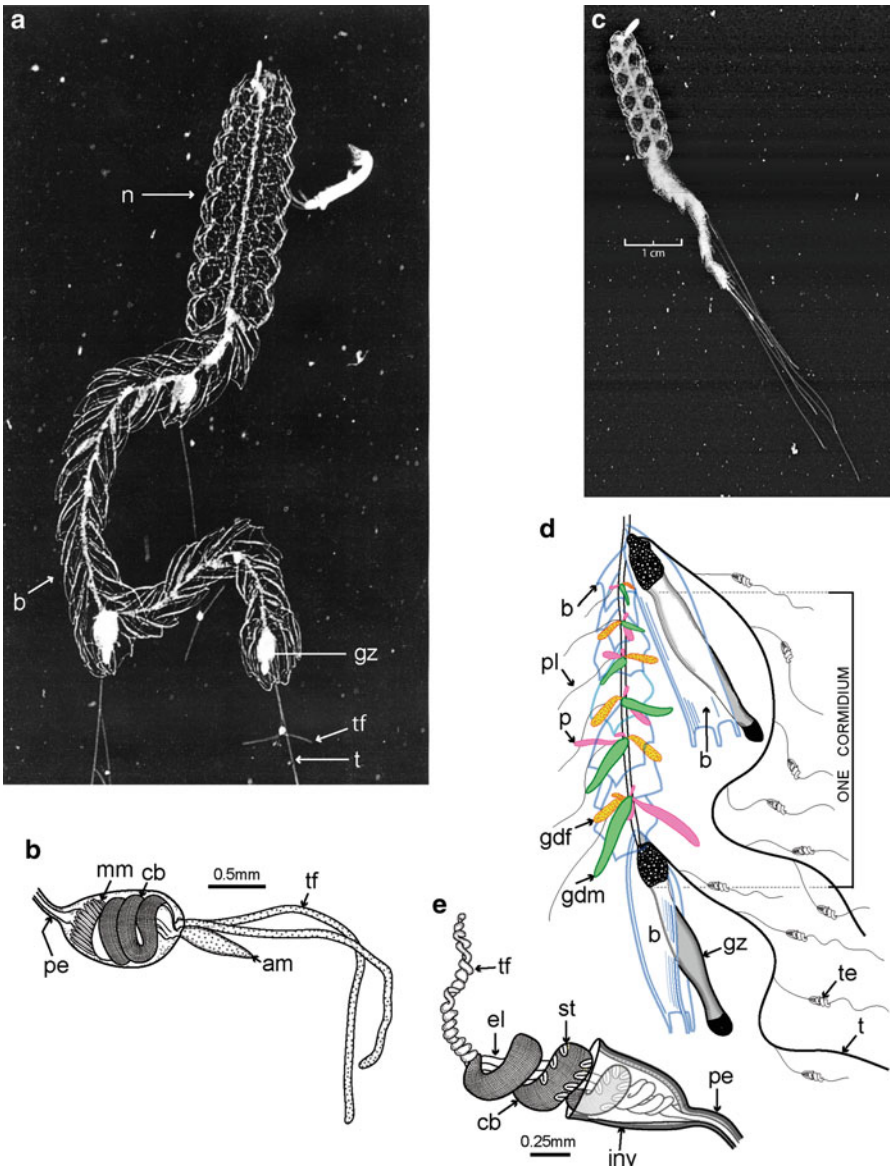

Fig. 12 Morphology of monoecious physonects 3. Two common species of the family Agalmatidae *sensu stricto*. (**a**) *Agalma elegans* (© Jessica Luo/Cowen Lab); (**b**) *A. elegans* tentillum (© Mapstone 2014, Fig. 13Aa); (**c**) *Nanomia bijuga* (© Inter-Research, revised from Luo et al. 2014); (**d**) *N. bijuga* cormidium; (**e**) *N. bijuga* tentillum (© Gillian Mapstone 2014, Figs. 6A, 13C). Labels: *am* ampulla, *b* bract, *cb* cnidoband, *el* elastic band, *gdf* female gonodendron, *gdm* male gonodendron, *gz* gastrozooid, *inv* involucrum, *mm* microbasic mastigophore, *n* nectophore, *pe* pedicel, *p* palpon, *pl* palpacle, *st* stenotele, *t* tentacle, *te* tentillum, *tf* terminal filament

Mapstone 2009, Fig. 19a). Pugh and Baxter (2014) also introduce a new species *H. maculatum* and redescribe *H. rubrum*, *H. cupulifera*, *H. striata*, *H. foliacea*, and *H. transliratum* from complete specimens, most collected in exceptionally good condition by submersible vehicles. Ridge patterns are clarified in both young and mature nectophores, bract types described (from two to five in this genus), and tentilla compared and contrasted. The cnidoband of *Halistemma* species is long (sometimes up to nine coils), with a very small cup or disk-shaped involucrum proximally and a single terminal filament distally; the latter often, but not always, terminates in a swollen acorn-shaped sinker. *Halistemma* tentilla are figured for all species by Pugh and Baxter (2014) and that of *H. transliratum* also shown by Mapstone (2014, Fig. 13b).

Species in the long-stemmed genera *Agalma* and *Nanomia* have yet to be described from submersible material, if indeed it exists, although both are well-known genera. They have differently shaped nectophores, one or two transparent bract types on the siphosome, and distinctively different tentilla (Fig. 12b, e). Their cnidobands tend to be shorter than those of *Halistemma* species and are red and in *Agalma* species completely covered in a transparent sac known as the involucrum (Fig. 12b), although in one rarer species (*Agalma clausi*) the involucrum is open distally and the bracts apparently bear distinctive red spots. *Agalma elegans* (Fig. 12a) is a soft and flexible species, uncommon but cosmopolitan in temperate and tropical latitudes (Mapstone 2014, Table 1). *A. okeni*, in contrast, is rigid, with a short stem bearing prismatic nectophores and bracts, and is quite often collected in warmer waters worldwide (Pugh 1999b).

Two short-stemmed genera *Athorybia* and *Melophysa* are in the Agalmatidae *sensu stricto* family (see Fig. 5 and Table 2) because *Athorybia rosacea* is sister to the three species in the genus *Agalma* (Fig. 3). *Athorybia rosacea* is a small species without any nectophores, which resembles a floating flower (Fig. 13a) and comprises a large pink pneumatophore surrounded by several whorls of large bracts arising from a much reduced corm-like siphosome (Fig. 13b). Gastrozooids each bear a tentacle with tentilla, which hangs down from the lower side of the corm for feeding, and in *A. rosacea* the tentilla are of two types: dendritic, without an involucrum and with dendritic growths (Fig. 13c*a*), and involucrate, with a complete involucrum when mature (Fig. 13c*c*) and a barely developed one when young (Fig. 13c*b*). Some dendritic tentilla also have a large protruding spur, which may act as a lure (Mapstone 2014, Fig. 16b). *Melophysa melo* (WoRMS Siphonophora List) differs from *A. rosacea* in having a short nectosome bearing up to four functional nectophores, but has similar tricornuate tentilla (with a terminal ampulla and two lateral terminal filaments) and a coiled cnidoband partly covered by an involucrum (Daniel 1985).

Totton (1965) concluded that *Athorybia rosacea* represents a young *Nanomia* or *Agalma* individual lying on its side, and this is because species of these two genera go through an *Athorybia* post-larval stage during their early development. Totton (1965) shows this stage in his Fig. 19 for *A. elegans*. The larva has a ring of larval bracts around the pneumatophore (but no nectophores) and a larval tentacle with a number of simple and different larval tentilla (shown for *Agalma elegans* in

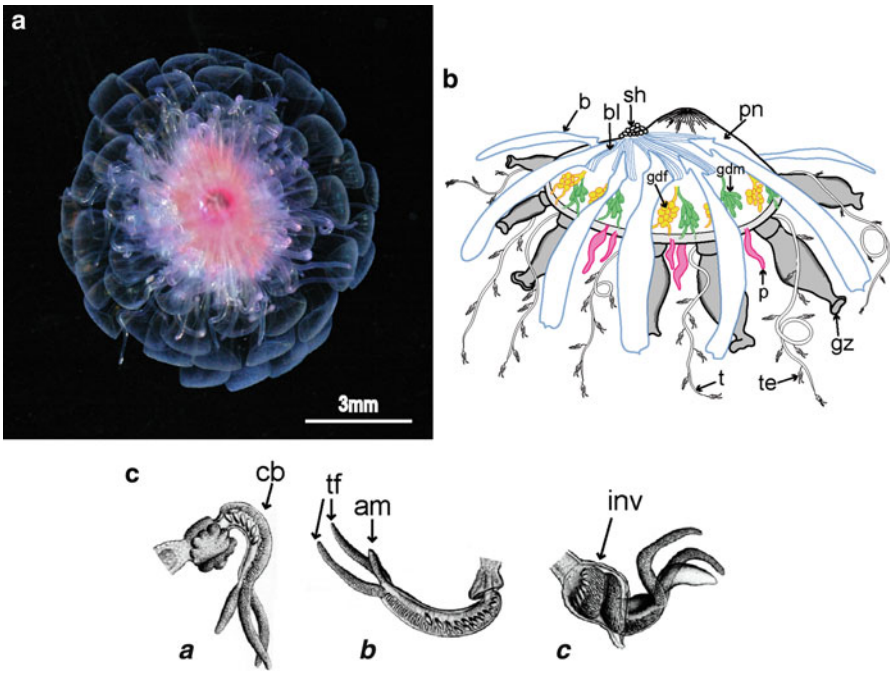

Fig. 13 Morphology of monoecious physonects 4: *Athorybia rosacea*. (**a**) Live individual floating in the sea, from above (© Larry Madin WHOI); (**b**) lateral view of float with siphosomal horn (where the cormidia are formed) and attached cormidia (© Gillian Mapstone 2014, Fig. 6D; see figure legend for reference from which the drawing derived); (**c**) ***a***, ***b*** dendritic tentilla and ***c*** involucrate tentillum (Bigelow 1911, pl. 20, Figs. 8, 9, and 10). Labels: *am* ampulla, *b* bract, *bl* bracteal lamella, *cb* cnidoband, *gdf* female gonodendron, *gdm* male gonodendron, *gz* gastrozooid, *inv* involucrum, *p* palpon, *pn* pneumatophore, *sh* siphosomal horn, *t* tentacle, *te* tentillum, *tf* terminal filament

Mapstone 2014, Fig. 13Ab). In *Halistemma* species, Pugh and Baxter (2014) have found a different developmental stage known as a *Nectalia* post-larva. This comprises a few nectophores and a ring of long larval bracts, with a larval tentacle bearing another type of larval tentillum, and has been shown so far for *H. rubrum* and *H. maculatum* (see Pugh and Baxter 2014, Figs. 23–25, 89, and 92), although is probably also found in the other *Halistemma* species. In addition, another *Nectalia* post-larva occurs in the dioecious physonect *Stephanomia amphytridis*, but in this species the larval tentilla are very different (see Pugh and Baxter 2014, Figs. 108, 111, and 112–113).

Unassigned Monoecious Genera

Three genera with ventral nectosomes are now excluded from the Agalmatidae *sensu stricto* and are listed in Fig. 5 and Table 3. Each is summarized briefly below from

Table 3 Characters for calycophoran families (Derived from © Mapstone 2014, Table 5; see this paper also for additional references omitted below; see Fig. 5 for details of fundamental siphonophore characters mentioned below)

	Family	Comments
14.	Prayidae	Probably paraphyletic and includes nested family Hippopodiidae (Dunn et al. 2005b) (see below); *Praya dubia* (subfamily Prayinae) and subfamily Nectopyramidinae may be one lineage, with prayines *Craseoa*, *Gymnopraia*, and *Rosacea* another (Dunn et al. 2005b), but broader taxa sampling is needed (Mapstone 2009). Prayine name *Lilyopsis medusa* has precedence over *Lilyopsis rosea* (WoRMS Siphonophora List); new prayine species *Desmophyes haematogaster*, *Gymnopraia lapislazula*, *Lilyopsis fluoracantha*, *Rosacea repanda*, *R. limbata*, and *R. arabiana* introduced (see WoRMS Siphonophora List); subfamily Nectopyramidinae revised with *Nectopyramis thetis* and *N. natans* redescribed and new genus *Nectadamas* introduced (for *N. diomedeae* and a new species *N. richardi*). Prayine species *R. cymbiformis* also redescribed and nomenclature problems concerning *R. plicata* sensu Bigelow and *Desmophyes annectens* resolved. Eudoxids released in amphicaryonines and nectopyramidines, but not in prayines (Mapstone 2009). *Rosacea villafrancae* transferred to genus *Desmophyes* and *Prayoides intermedia* found to be a junior synonym of *Praya* species (Pugh 1992, WoRMS Siphonophora List). Unique bio-optical properties identified in *G. lapislazula* and *L. fluoracantha*, though their function is still unknown (Haddock et al. 2005a)
15.	Hippopodiidae	Found nested within prayines in first siphonophore phylogeny and *Hippopodius* nested within *Vogtia* (Dunn et al. 2005b); hippopodiid distribution correlated with feeding on various species of ostracods, unlike other calycophorans. Family characters recently summarized and the new axes applied, together with redescriptions given and synonomies listed for *V. serrata*, *V. spinosa*, and *V. pentacantha* (Mapstone 2009); *V. microsticella* considered a junior synonym of *V. glabra* and *V. kuruae* a junior synonym of *V. serrata* (WoRMS Siphonophora List; Mapstone 2009)
16.	Clausophyidae	The three diphyomorph families below may have arisen from this one (Dunn et al. 2005b). New species include *Clausophyes laetmata* and *Cl. tropica*, and two others redescribed include *Cl. galeata* and *Cl. moserae*; a unique fuseudoxid life stage found in *Crystallophyes amygdalina* and a new genus *Kephyes* introduced for Moser's *Cl. ovata*, which, unlike *Clausophyes* species, has bracts with a pair of hydroecial canals (Pugh 2006b). Four clausophyids redescribed from NE Pacific and new axes applied (Mapstone 2009)
17.	Sphaeronectidae	Ten species now considered valid in this family with single retained larval nectophore. Family reviewed and history summarized (Pugh 2009); five new species introduced: *Sphaeronectes christiansonae*, *S. haddocki*, *S. tiburonae* (Pugh 2009), *S. pagesi*, and *S. pughi*. An old species *S. brevitruncata* reinstated (Pugh 2009) and *S. bougisi* concluded to be a likely calyconula of *Lilyopsis medusa* (WoRMS Siphonophora List). *S. gracilis* relegated to a junior synonym of *S. koellikeri* and probably restricted to the tropics (Pugh 2009; WoRMS Siphonophora List); specimens reported from Jervis Inlet, British Columbia (Mapstone 2009), could be *S. haddocki*

(*continued*)

Table 3 (continued)

	Family	Comments
18.	Diphyidae	Probably paraphyletic (Dunn et al. 2005b), vindicating earlier conclusions (Totton 1965), but based on only 5 of 45 likely valid species (WoRMS Siphonophora List). Two main clades identified in the molecular study, within one of which is nested the family Abylidae (Dunn et al. 2005b). New axes applied to all life stages of diphyids; muscular lamellae, median gastrovascular canals, and pedicular canal arrangements also schematically shown for two basic types of diphyids (Mapstone 2009). A new small species added to the genus *Lensia* (*L. quadriculata*), another redescribed in detail (*L. asymmetrica*), and a third (*L. reticulata*) transferred to a new genus *Gilia* within a new subfamily Giliinae, for the two clausophyid-like canals in the bract. An enigmatic species *Eudoxia macra* shown, using the mitochondrial 16S gene, to be sexual stage of a larger species *L. cossack*. A number of previously described *Lensia* species, several *Sulculeolaria* species, and one *Muggiaea* species all reduced to junior synonyms of various better known species (WoRMS Siphonophora List)
19.	Abylidae	Family nested with *Diphyes dispar* in one of two Diphyidae clades, based on 16S and 18S (Dunn et al. 2005b), but only *Abylopsis tetragona* tested and more taxa sampling needed. Ten valid species (WoRMS Siphonophora List), all present in the South Atlantic and summarized by Pugh (1999b); several species also redescribed from around South Africa. Junior synonyms (including those in a confusing abylid review by Sears) given in the WoRMS Siphonophora List

information given by Mapstone (2009, 2014) and references quoted therein. *Cordagalma ordinata* is a flexible species up to 30 cm long with a maximum of 40 diminutive heart-shaped nectophores, distinctive kite-shaped bracts, palpons without palpacles, and unique larval-type tentilla on the gastrozooid tentacles which are very small and lack a cnidoband (Mapstone 2014, Fig. 13d). *C. ordinata* feeds only on small copepods (Purcell 1980), which are trapped in an array of long cnidocils that project from the distal surface of the tentillum. The species inhabits all oceans and can sometimes be abundant in deep coastal fjords. It has been collected by submersible in the Alboran Sea in the western Mediterranean and studied in detail at Villefranche Marine Station. These and other worldwide records are summarized in Mapstone (2009).

Frillagalma vityazi is a small physonect with larger nectophores than those of *Cordagalma ordinata,* but a shorter and rigid siphosome. Nectophores bear pairs of ridges similar to those found in other physonects (including a single pair of vertical-lateral ridges), a nectosac with simple looped lateral radial canals and bioluminescent patches on the nectophore surface. Bracts are facetted and of three types, with three pairs per cormidium and, as in *C. ordinata,* the palpons lack palpacles and the tentilla are distinctive and diagnostic. Each tentillum comprises a very small proximal cnidosac with c. 33 nematocysts of two types, from which project two elongate and sausage-shaped sequential ampullae (Mapstone 2014, Fig. 13e). *F. vityazi* is a

rare species worldwide (distribution summarized in Mapstone 2009) and has been collected with submersibles in the Bahamas, but prey consumed is so far unknown.

The third monotypic species in this group is *Lychnagalma utricularia*. It is very fragile and transparent and has only rarely been collected worldwide (WoRMS Siphonophora List), with most specimens coming again from submersible dives in the Bahamas region (Pugh and Harbison 1986). *L. utricularia* shares certain characters with Agalmatidae *sensu stricto* species, including nectophores with paired ridges and the tentilla which are involucrate with a long coiled cnidoband, but differs in having a ventral nectosome. Unusually for a physonect, it is completely non-bioluminescent, and the mature tentillum is also exceptionally large, reaching up to 7.5 mm in length (see Mapstone 2014, Fig. 16c), with a large central ampulla and eight terminal filaments. These pulsate like a swimming medusa and form an intriguing lure which may perhaps attract small fish, although so far no prey has been found in any of the gastrozooids collected from *L. utricularia*.

Family Prayidae

The Calycophorae is a monophyletic clade (Fig. 3) which is monoecious, has lost the pneumatophore (Fig. 5), and retained reduced larval cormidia (Dunn and Wagner 2006). Species in the six calycophoran families (Table 3) have only two nectophores (sometimes one and occasionally four or more) which are alike and apposed in prayomorphs (Fig. 14b, e) and different and linearly aligned in diphyomorphs; they also lack the axial wings and thrust block of physonect nectophores. A single larval nectophore develops from the calyconula larva, before the first definitive nectophore appears. Prayidae is one of the largest calycophoran families, including 27 species (Fig. 4), and systematic changes since 1987 are summarized in Table 3. In life prayomorph nectophores attach at the anterior end of a typically very long siphosome bearing hundreds of cormidia (Fig. 14a). When feeding a "sit and wait" strategy is employed, when the stem relaxes and the extended tentacles hang down in a long feeding curtain.

The largest of the three subfamilies is the Prayinae, which undergo nectophore replacement, probably throughout life. The first definitive nectophore develops inside a long proximal groove in the larval nectophore (termed the hydroecium) and matures (Pugh 1992), and then a second definitive nectophore may start to form before the larval nectophore is shed. Buds for third and fourth nectophores are often also visible inside the hydroecium, and these will enlarge and replace earlier definitive nectophores over time, as summarized by Mapstone (2009). Stem cormidia are retained throughout life in prayines and comprise a single rounded bract (except *Gymnopraia*) with typically six bracteal canals enclosing a gastrozooid, tentacle, and gonophore (Fig. 14c). Prayine tentilla are all alike (Fig. 14d*a*) with a swollen sinker at the distal end of the terminal filament to act as a weight (Fig. 14d*b*). Unlike physonects, tentilla are not useful for species or genus diagnosis in calycophorans, and prayine species are separated on nectophore and bract characters. These include the relative size of nectosac to nectophore (small in

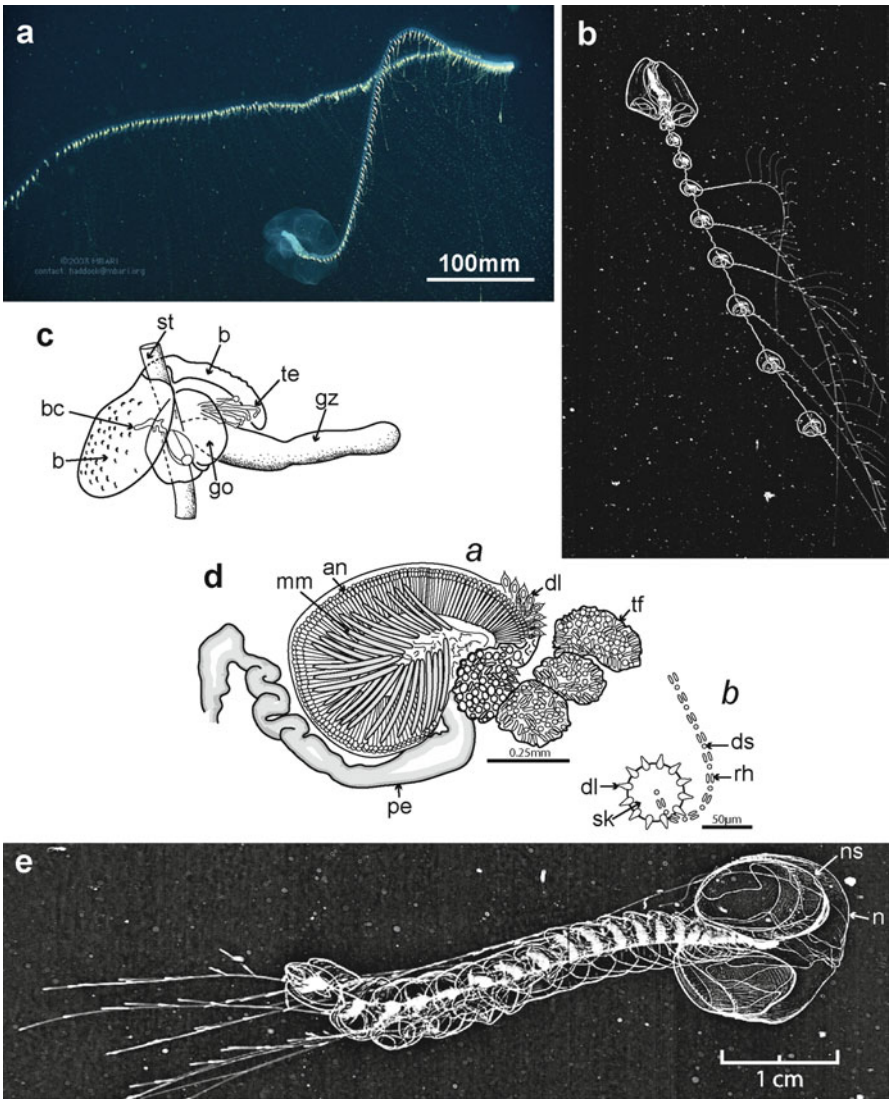

Fig. 14 Prayine prayomorph morphology. (**a**) Typical prayomorph *Praya* sp. feeding, with two rounded bells and a very long siphosome bearing over 100 cormidia; tentacles extended for feeding, each bearing 80–90 nematocyst batteries, giving <9,000+ batteries in all (Steven Haddock © MBARI). (**b**) *Rosacea* sp. feeding (© Jessica Luo/Cowen Lab); (**c**, **d**) *Rosacea cymbiformis*; (**c**) cormidium enlarged; (**d**) ***a*** tentillum, ***b*** sinker (© Gillian Mapstone 2014, Figs. 8A and 14A); (**e**) *Lilyopsis rosea* swimming (© Jessica Luo/Cowen Lab). Labels: *ani* anisorhizas, *b* bract, *bc* bracteal canal, *c* cormidium, *dl* large desmoneme, *ds* small desmoneme, *go* gonophore, *gz* gastrozooid, *mm* microbasic mastigophore, *n* nectophore, *ns* nectosac, *rh* rhopalonemes, *pe* pedicel, *sk* sinker, *st* stem, *t* tentacle, *te* tentilla, *tf* terminal filament

Praya and *Rosacea* species (see Fig. 14b) and large in *Lilyopsis* (see Fig. 14e)), presence or absence of a disjunct pedicular canal (see Mapstone 2009, Fig. 5), the type and branching of the somatocyst when present, the branching and courses of the lateral radial canals of the nectophore, and the number and arrangement of canals in the bract (all summarized in Mapstone 2009).

Prayine nectophores vary in size, with some species reaching as much as 6 or 10 cm in length (*Praya* species and *Rosacea cymbiformis*), although most are shorter (circa 3–4 cm) and others diminutive (*Mistoprayina fragosa*, *Prayola* species, *Rosacea arabiana*, 3–6 mm in length (WoRMS Siphonophora List)). Prayines with large nectosacs are very fragile and hence rarely successfully collected (*Lilyopsis* Fig. 14e), while others with more mesogloea are robust (*Praya* and *Rosacea* Fig. 14a, b) and collected periodically in epi- and mesopelagic waters of most seas (*Praya dubia* and *Rosacea plicata*; see Mapstone 2014, Table 1; also *R. cymbiformis* Mapstone 2009).

The two much smaller prayid subfamilies Amphicaryoninae and Nectopyramidinae have only four species apiece and are probably derived from the prayines, although only two species were included in the molecular analysis of Dunn et al. (see Fig. 3). Amphicaryonines are small, rounded, and composed of two unequal-sized nectophores: a larger retained larval nectophore and a smaller reduced definitive nectophore (see summary in Mapstone 2009). The nectosac is functional in both nectophores of the largest amphicaryonine *Maresearsia praeclara* (20 mm diameter), but only in the larval nectophore of the three smaller *Amphicaryon* species (WoRMS Siphonophora List). The best known amphicaryonine is *A. acaule* (8 mm diameter) (Fig. 15a), which inhabits epi- and mesopelagic layers of warmer waters worldwide (see Mapstone 2014, Table 1). All amphicaryonines release small free-living eudoxids with only two canals in the bract, for species dispersal.

Nectopyramidine prayids develop only one ridged asymmetric definitive nectophore, from a smaller ridged larval nectophore, and also release a free-living eudoxid (Fig. 15b) (reviewed by Mapstone 2009). Definitive nectophores vary from pyramidal to rhomboidal or bow-shaped, with a penetrating somatocyst of one or several branches and a hydroecium of varied shape. This subfamily is rare worldwide, being mainly mesopelagic with a greater latitudinal range than amphicaryonines (though absent from the Mediterranean) (Mapstone 2009, 2014). Nectophores and eudoxids of three of the four species are large, reaching up to 36 mm or more in length.

Family Hippopodiidae

Hippopodiidae is a small and unusual calycophoran family of five species each with up to 12 nectophores (Fig. 16a–c, e) and found to be nested with the family Prayidae by Dunn et al. (2005b) (Fig. 3, Table 3). Like prayine prayids, the first definitive nectophore develops inside the hydroecium of a small rounded larval nectophore (see Mapstone 2009, Fig. 42), which is later shed and more definitive nectophores formed, each from the pedicel of its predecessor. Thus, the largest nectophore, and

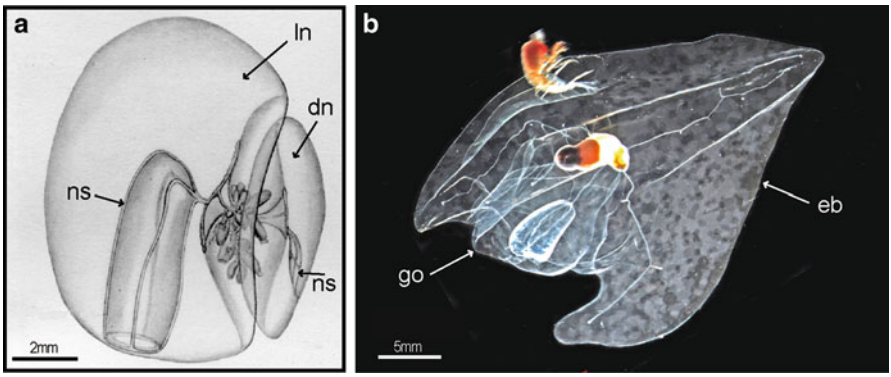

Fig. 15 Amphicaryonine and nectopyramidine prayomorph morphology. (**a**) *Amphicaryon acaule* colony (Bigelow 1911, pl. 4 Fig. 1); (**b**) *Nectadamas diomedeae* eudoxid (© Russ Hopcroft UAF). Labels: *dn* definitive nectophore, *eb* eudoxid bract, *go* gonophore, *ln* larval nectophore, *ns* nectosac

the only one with a functional nectosac, occurs at the base (or posterior end) of the colony (Fig. 16b). There are no bracts in the siphosomal cormidia (Fig. 16d), and this allows the stem to be more easily withdrawn into the chamber created by the nectophores (Fig. 16a, c, e). Buoyancy for the colony is instead provided by the thick and typically robust nectophores, which are either rounded with two or more protuberances on the distal side of the nectosac (*Hippopodius hippopus* and *Vogtia glabra*; see Mapstone 2009, Fig. 2f and g for nectophore axes) or angular and pentagonal, often with ridges and cusps (Mapstone 2009). Buoyancy in hippopodiids is likely controlled by active transport of lighter and heavier ions across the covering epithelium (Mackie and Mackie 1967).

Although two genera are included within the family Hippopodiidae, *Hippopodius* and *Vogtia* (WoRMS Siphonophora List), the differences between them are small. Both genus names are very old and are still retained only to avoid confusion in the literature (Totton 1965). All five species have nectophores of similar dimensions (<21 mm along upper-lower axis) and a widespread cosmopolitan distribution. However, they occur at varied latitudes and depth horizons, and all feed selectively on ostracods (Table 3), unlike all other calycophorans.

Hippopodius hippopus is the most abundant and best known species. It has tentilla with a very long terminal filament for feeding (Fig. 16f) and is a robust and epipelagic species, which lives in warmer waters worldwide (Mapstone 2014), often occurring nearer the coast than the four *Vogtia* species. It undergoes blanching, and this, together with nerve/epithelial conduction, has been studied in detail by Mackie (reviewed in Mackie et al. 1987). *Vogtia glabra* is the only rounded *Vogtia* species (Fig. 16e), with just two prominences distal of the ostium when mature (Pugh 1999b). *V. glabra* also prefers, like *H. hippopus*, tropical and temperate waters, but differs in inhabiting mainly the mesopelagic zone, with many fewer and sporadic records from the world's oceans.

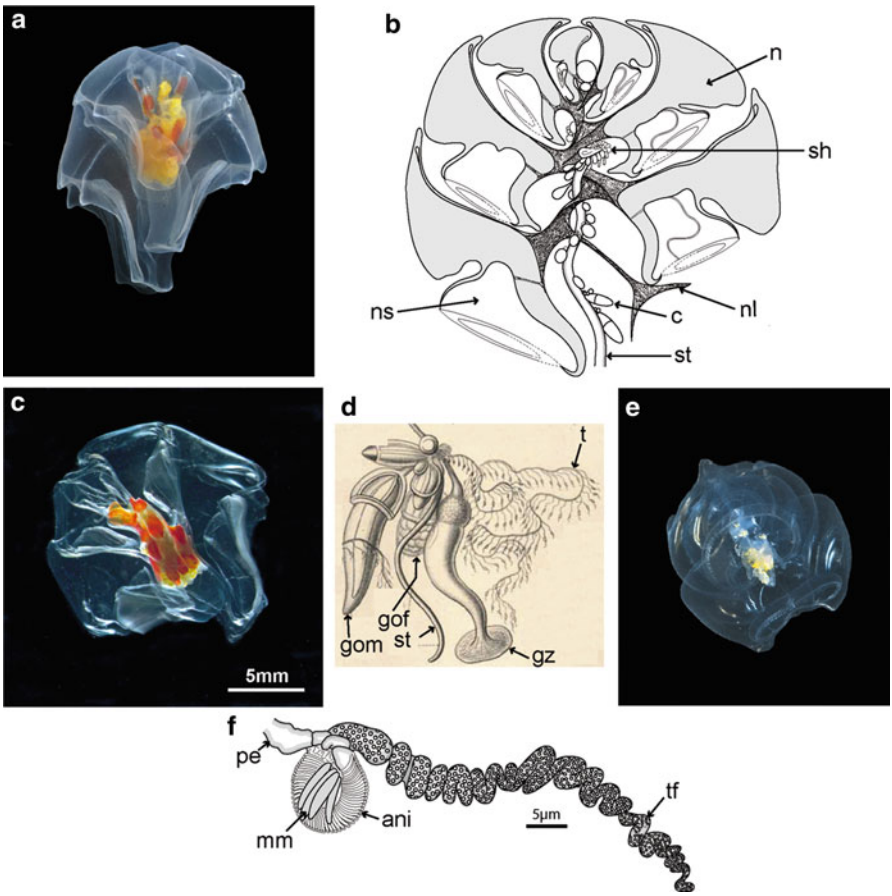

Fig. 16 Hippopodiid prayomorph morphology. (**a**) *Hippopodius hippopus* colony (© Sonke Johnsen, Duke); (**b**) section through *Hippopodius* (© Gillian Mapstone 2014, Fig. 8B; see figure legend for original reference); (**c**) *Hippopodius hippopus* colony (© Russ Hopcroft UAF); (**d**) *Hippopodius hippopus* cormidium; note, no bracts (© Mapstone 2014, Fig. 8D; see figure legend for original reference); (**e**) *Vogtia glabra* colony (© Sonke Johnsen, Duke); (**f**) *Hippopodius hippopus* tentillum (© Gillian Mapstone 2014, Fig. 14C; see figure legend for original reference). Labels: *ani* anisorhizas, *c* bractless cormidium, *gof* female gonophore, *gom* male gonophore, *gz* gastrozooid, *mm* microbasic mastigophore, *n* nectophore, *nl* nectophoral lamella, *ns* nectosac, *pe* pedicel, *sh* siphosomal horn, *st* stem, *t* tentacle, *tf* terminal filament

Of the three pentagonal *Vogtia* species, *V. serrata* is the largest and the most abundant (Mapstone 2009), with an extensive latitudinal range in both hemispheres (Mapstone 2014, Table 1), inhabiting shallow depths in the Antarctic, and deeper layers in temperate seas, where it is typically the dominant hippopodiid of mesopelagic assemblages, and also the deepest living (reviewed by Mapstone 2009). *V. pentacantha* is a smaller and less frequently encountered *Vogtia*, with cusped ridges but smooth facets on the nectophores, and is also mainly mesopelagic. In

contrast, nectophores of *V. spinosa* have cusps on both the facets and the ridges, and this species is epipelagic at lower latitudes and mesopelagic at higher latitudes (Mapstone 2009).

Family Clausophyidae

The remaining four calycophoran families in Table 3 are all diphyomorphs, which typically have two dissimilar angular and also often streamlined nectophores strengthened with longitudinal ridges and containing a relatively large powerful nectosac and little mesogloea; they also contain a swollen blind-ending diverticulum from the gastrovascular canal system termed the somatocyst (Fig. 2c) which is mirrored in the canal system of the eudoxid bract as a swollen phyllocyst. These structures might act as food stores and/or increase buoyancy (Mapstone 2009).

Clausophyidae is a small family of ten species which are mostly meso- and bathypelagic and hard to sample. They were poorly understood for many years until the advent of modern sampling methods. Clausophyids were only raised to family status in 1965, in contrast to the other calycophoran families which are much older (Mapstone 2009, 2014; Totton 1965, WoRMS Siphonophora List). Distinctive family characters include typically two nectophores, both containing somatocysts and with the posterior larger than the anterior, aligned partially linearly, and partially in apposition (Mapstone 2009, Fig. 4). This latter character suggests that they may represent the ancestors of the other diphyomorph families, as noted in Table 3. Cormidia are released as free eudoxids in three of the five genera (*Chuniphyes*, *Kephyes*, and *Heteropyramis*), but bracts are absent in *Clausophyes* species, and each bract is fused with a gonophore in the monotypic genus *Crystallophyes* (Table 3). Few clausophyid species were sampled in the molecular analysis of Dunn et al. (2005b) (Fig. 3), and, although the results indicate that this family might be paraphyletic, the nodes are poorly supported and further sampling is needed.

There is considerable size variation among clausophyid genera, with anterior nectophores of *Chuniphyes* growing up to 30 mm in length, while those of *Heteropyramis* (which does not develop a posterior nectophore) reaching only 5 mm; nectophores of *Clausophyes* and *Kephyes* are of intermediate size (Fig. 1; see Pugh (2006b) and Mapstone (2009) for further details and other references). The somatocyst reaches to the anterior end of both nectophores when mature, and in the anterior nectophore the nectosac typically extends to only half its length (Fig. 17b, c; *K. ovata* in Fig. 17a is an exception). The stem attaches to the hydroecial wall of the anterior nectophore some distance anterior of the ostium (Fig. 17b), suggesting that clausophyids might be an intermediate stage in the migration of the posterior nectophore from the apposed position in prayids to the superimposed, or linearly aligned, position in most Diphyidae and Abylidae (see Mapstone 2009, Fig. 4). The many nomenclatural problems among some species of the family Clausophyidae have been resolved in recent years, as discussed by Mapstone (2009).

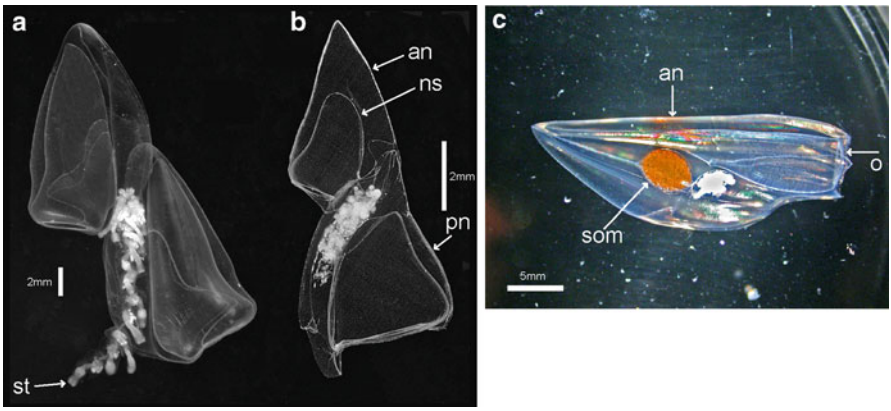

Fig. 17 Clausophyid diphyomorph morphology. (**a**) *Kephyes ovata* and (**b**) *Clausophyes moserae* (both © JMBA Plymouth, from Pugh 2006b); (**c**) *Chuniphyes multidentata* anterior nectophore only (posterior nectophore detached during capture) (© Casey Dunn). Labels: *an* anterior nectophore, *ns* nectosac, *o* ostium, *pn* posterior nectophore, *som* somatocyst, *st* stem

Clausophyids are mostly cosmopolitan worldwide (see Mapstone 2014, Table 2), but none are common, and, indeed, species such as *Clausophyes laetmata*, *C. galeata*, and *C. tropica* are rare to very rare (WoRMS Siphonophora List). Perhaps the most successful clausophyid is the large species *Chuniphyes multidentata* which has a considerable latitudinal range worldwide and produces a large number of small eudoxids when breeding (Mapstone 2009). Although it inhabits the same depth horizons as its congener *C. moserae*, Mackie et al. (1987) conclude that these two species are allopatric in the North Atlantic at least, with *C. moserae* being more prevalent below 40°N and *C. multidentata* more abundant above this latitude; where the two species coexist, their population nuclei are spread over different depth zones (summarized in Mapstone 2009).

Family Sphaeronectidae

Sphaeronectidae is a small and distinctive family of ten valid species and one *species inquirenda* (WoRMS Siphonophora List), in which a rounded larval nectophore is retained into adulthood (paedomorphy), producing cormidia on the elongate siphosomal stem which are released into the plankton as free-living eudoxids, like diphyids and abylids (Mapstone 2009). No definitive nectophores are formed. Only one species was sequenced by Dunn et al. (2005b), appearing in their molecular phylogeny as *Sphaeronectes gracilis* (Fig. 3); this species has since been referred to *S. koellikeri* (Mapstone 2009; Pugh 2009). The species is firmly nested within the diphyomorph clade of calycophorans (Fig. 3), dispelling some earlier ideas about affinities of sphaeronectids. All sphaeronectid species are small, with a single

rounded or ovoid nectophore that is very fragile and varies in size from 1.5 to 11.5 mm (Pugh 2009). As a result, most plankton nets miss these small calycophorans, and several new species have been discovered recently by SCUBA divers and using fine-meshed nets deployed in the surface layers of coastal waters around various continents. Despite this, two of the newly introduced species are meso- and bathypelagic, found only so far in Monterey Bay (Pugh 2009, Fig. 18).

Axes for nectophores of *Sphaeronectes koellikeri* are given by Mapstone (2009, Fig. 3g), and all species have a small hydroecial opening on the lower nectophore surface, since no second nectophore has to be accommodated (Fig. 18a–c). All species except *S. koellikeri* also have a small and very short hydroecium which originates on the lower side of the nectosac; in *S. koellikeri*, the hydroecium uniquely extends over the top of the nectosac on the anterior side (Mapstone 2009, Fig. 65a). Species differ in the ratio of nectosac to nectophore length, position of origin of the four radial canals on the nectosac, and the courses of the lateral radial canals over the nectosac to the ostial ring canal. Size, shape, color, extent, and position of the somatocyst are also important for species identification, with some having an elongate tubular somatocyst, most having a pyriform one, and one species having

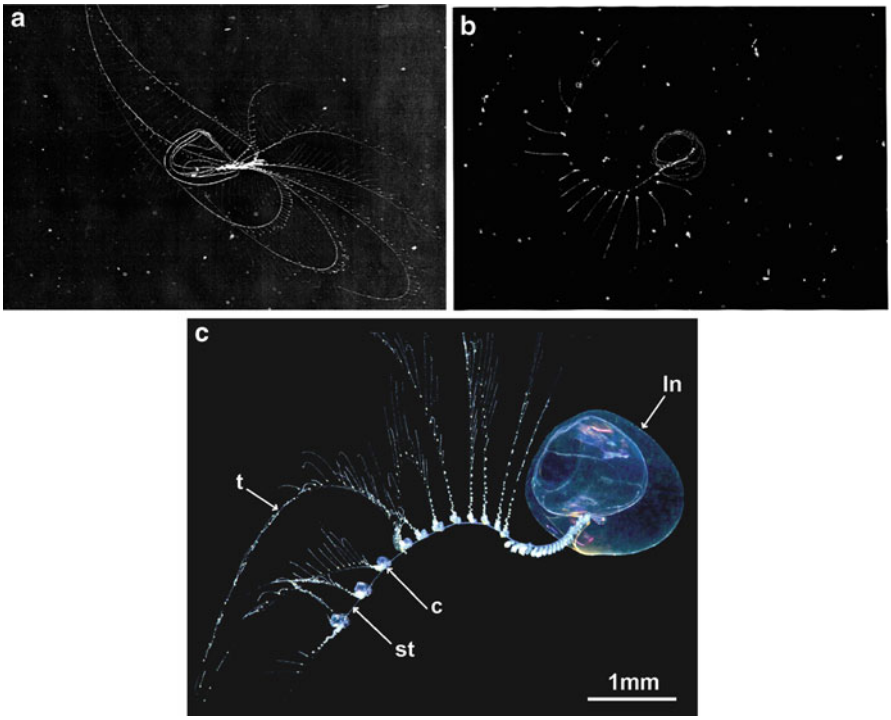

Fig. 18 Sphaeronectidae diphyomorph morphology. (**a**, **b**) *Sphaeronectes* spp. feeding (© Jessica Luo/Cowen Lab); (**c**) *Sphaeronectes pagesi* feeding (© D. Lindsay, R. Minemizu, JAMSTEC). Labels: *c* cormidium, *ln* larval nectophore, *st* stem, *t* tentacle

a stalked somatocyst. In *S. tiburonae* from Monterey Bay, the somatocyst is minute, and in *S. christiansonae*, also from this location, the somatocyst is red (Pugh 2009). Eudoxids are so far known for only three of the ten valid species, and all differ in phyllocyst shape and bract to gonophore ratios.

Only one sphaeronectid species, *Sphaeronectes koellikeri,* is well known and its distribution, together with those of five of the other species, is summarized by Pugh (2009). Two species more recently introduced are noted in Table 3, and their distributions are limited so far to Japanese waters and subantarctic waters off Australia (see Mapstone 2014 for references). The identity of another small species originally referred to the genus *Sphaeronectes* is now likely a calyconula of *Lilyopsis medusa* (Table 3).

Family Diphyidae

Diphyidae are the most successful and speciose siphonophore family (Fig. 4), currently comprising 45 species considered valid (WoRMS Siphonophora List). Diphyidae dominate surface layers in the ocean, and their systematics is very stable because most species were introduced many years ago. Recent changes are summarized in Table 3, and Fig. 3 shows that in the molecular phylogeny, abylids (represented by *Abylopsis tetragona*) are nested within the five diphyid species tested.

Diphyids typically have two linearly aligned nectophores, led by a pointed streamlined anterior nectophore, and followed by a smaller posterior nectophore (Fig. 19a–c). The stem is completely withdrawn into the elongate hydroecium of the posterior nectophore for swimming, which alternates with a motionless phase during which the stem and tentacles relax and form a fishing net for feeding (Mapstone 2009, p. 30) (Fig. 19a, c). Nectophores are typically ridged and have a nectosac which fills the nectophore, a mouthplate adjacent to the ostium, and, in the anterior nectophore only, a discrete somatocyst food storage/buoyancy organ (Mapstone 2009, Fig. 3d). The stem bears numerous cormidia which each comprise a bract, gastrozooid and tentacle (Fig. 19d), and, when mature, a gonophore for reproduction. The structure of diphyid-type tentilla and their nematocyst compliments are summarized by Mapstone (2014, Table 8, Fig. 14d, e), and tentillum structure is shown for three diphyomorph species in Figs. 19e and 20c, f. The typical bract is typically helmet shaped (Fig. 20b) with a food storage equivalent of the somatocyst, the phyllocyst, and no bracteal canals. The gonophore has a large nectosac for propulsion, and, when released into the plankton as a free-living eudoxid from the posterior end of the stem, can live for several months and release a large number of gonophores for sexual reproduction.

The family includes three subfamilies: the Sulculeolariinae (five species) in which the stem is very long because cormidia are never released and nectophores can be replaced up to four times during life, the Diphyinae (39 species) with typically two ridged nectophores which cannot be replaced and a shorter stem from which eudoxids are released when mature, and the Giliinae (summarized in Table 3).

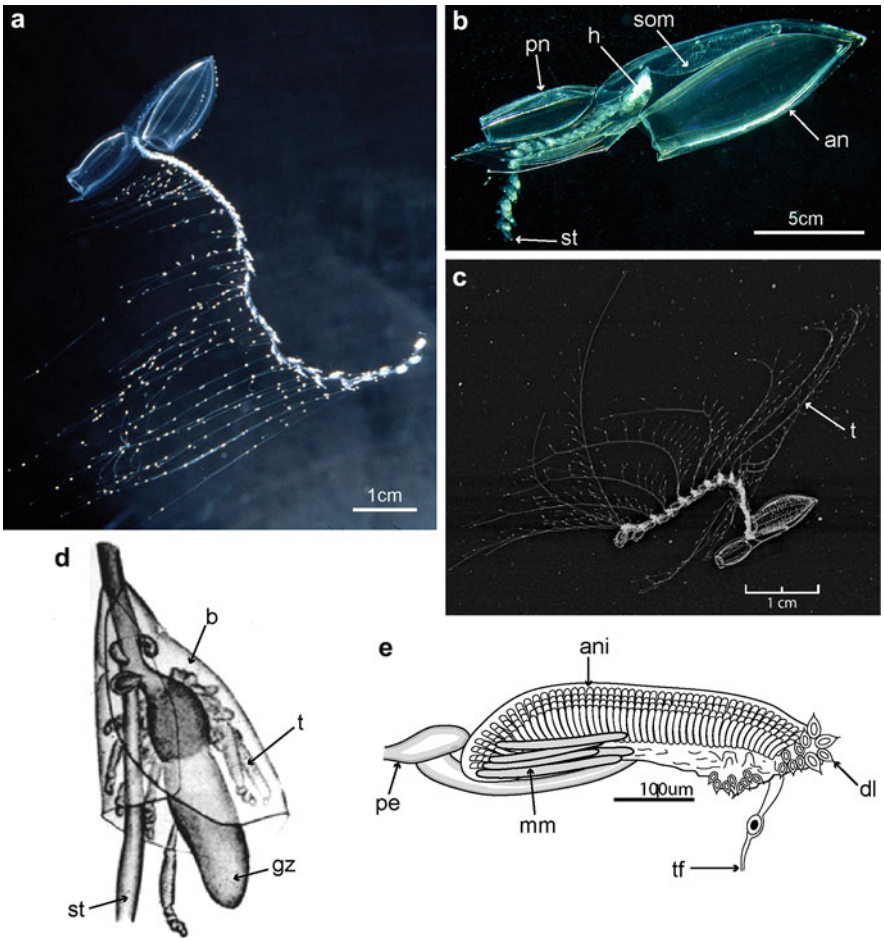

Fig. 19 Diphyid diphyomorph morphology. (**a**) *Lensia conoidea* feeding (Rob Sherlock © MBARI); (**b**) *Chelophyes appendiculata* (© Peter Schuchert MHNG); (**c**) *Lensia multicristata* feeding (© Inter-Research, revised from Luo et al. 2014); (**d**) *C. appendiculata* cormidium; and (**e**) *Diphyes dispar* tentillum (© Gillian Mapstone 2014, Figs. 8C and 14D; see figure legends for original references from which these figures were derived). Labels: *an* anterior nectophore, *ani* anisorhizas, *b* bract, *dl* large desmonemes, *gz* gastrozooid, *h* hydroecium, *mm* microbasic mastigophores, *pe* pedicel, *pn* posterior nectophore, *som* somatocyst, *st* stem, *t* tentacle, *tf* terminal filament

Sulculeolariines are warm-water epipelagic and cosmopolitan species (Mapstone 2014, Table 2), separated on the length of the somatocyst, the presence or absence of teeth around the ostium and small swellings on the upper sides of the mouthplate (Mapstone 2009, Fig. 44). Anterior nectophores vary in size from 8 to 26 mm, with *S. quadrivalvis* being the largest and probably most abundant species worldwide (Totton 1965).

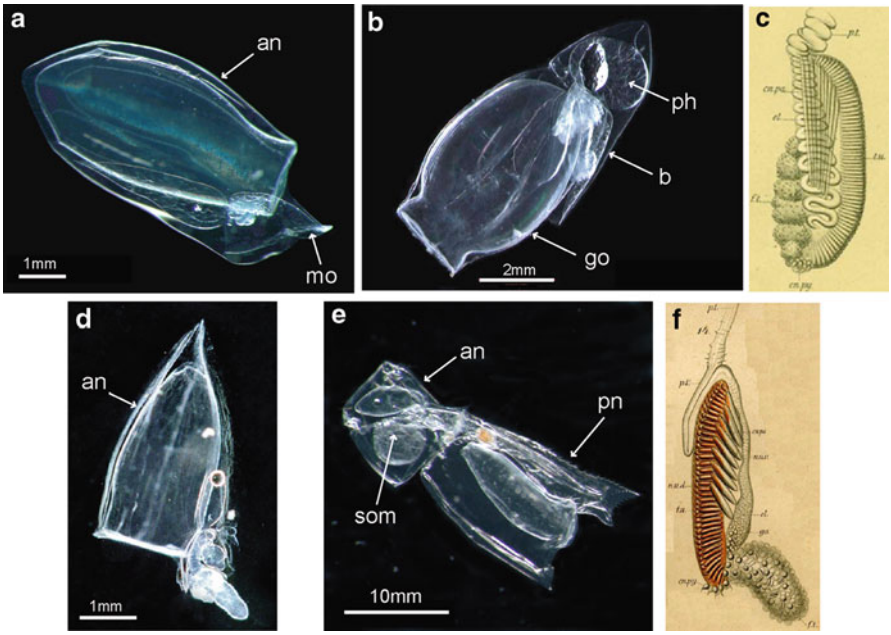

Fig. 20 Diphyid and abylid diphyomorph morphology. (**a**, **b**) *Dimophyes arctica* polygastric colony (**a**) and eudoxid (**b**) (© Russ Hopcroft UAF); (**c**) *D. arctica* tentillum (Chun 1897, pl. 1 Fig. 9); (**d**) *Muggiaea kochi* (© Peter Schuchert MHNG); (**e**) *Abylopsis tetragona* (© Russ Hopcroft UAF); (**f**) *Enneagonum hyalinum* tentillum (Chun 1892, pl. 12 Fig. 14). Labels: *an* anterior nectophore, *b* bract, *go* gonophore, *mo* mouthplate, *ph* phyllocyst, *pn* posterior nectophore, *som* somatocyst

Diphyines include 39 valid species in five genera (WoRMS Siphonophora List), which are separated on characters of the anterior nectophore, and in a few species a posterior nectophore does not develop (Fig. 20d). The genus *Chelophyes* comprises two distinctive warm-water 5-ridged epipelagic species with a claw-shaped hydroecium (Fig. 19b) and allopatric distribution. *C. appendiculata* is the most abundant siphonophore species worldwide, with an anterior nectophore more than twice the size of *C. contorta* and a much broader latitudinal range (Mapstone 2009, 2014; Pugh 1999b). *Dimophyes arctica* is monotypic for the genus *Dimophyes*, has a smooth anterior nectophore with a prominent undivided mouthplate (Fig. 20a), a very small posterior nectophore rarely collected, and a particularly broad cosmopolitan distribution, occupying deeper layers at lower latitudes and surface layers at higher ones (Mapstone 2009, 2014, Table 2). *Diphyes* anterior nectophores have a deep hydroecium, prominent teeth around the ostium, are all epipelagic, and include the largest of all diphyid species, *D. dispar* (<36 mm long, Pugh 1999b). Three of the four species are tropical, with two common worldwide (*D. dispar* and the much smaller *D. bojani*) and the third, which lacks a posterior nectophore, inhabiting only the Indo-Pacific region (*D. chamissonis*) (Totton 1965). The fourth *Diphyes* species,

D. antarctica, is also large (<30 mm long) and common, but only in the cold waters of the Southern Ocean (Pugh 1999b).

Two more small tropical diphyines are referable to the genus *Eudoxoides* and have an anterior nectophore with five serrated ridges, a hydroecium reaching 1/3 nectophore length (from the ostium), and a prominent mouthplate (Pugh 1999b). *E. mitra* is common and epipelagic in all oceans except the Mediterranean (Mapstone 2014, Table 2), while *E. spiralis*, which lacks a posterior nectophore, has a slightly more extended vertical and latitudinal distribution (Pugh 1999b). The genus *Muggiaea* (Fig. 20d) includes four small species (4–10 mm long) without a posterior nectophore, so the stem is accommodated during swimming inside a deep hydroecium in the anterior nectophore. Three species are neritic, restricted to the shallow shelf waters fringing continents, with *M. atlantica* occupying temperate waters worldwide, *M. kochi* replacing it in tropical Atlantic waters, and *M. delsmani* in tropical Indo-Pacific waters; these temperate/tropical pairs can also coexist, for example, in the English Channel (Mapstone 2009) and Sagami Bay (Grossmann and Lindsay 2013). The fourth species, *M. bargmannae*, is a bipolar species living only at very high latitudes in epi- and mesopelagic layers (Mapstone 2014). *M. delsmani* from the South China Sea was unfortunately misidentified by Lo et al. (2012) as *M. kochi*.

Lensia is a catch-all diphyine genus of circa 26 diverse valid species (WoRMS Siphonophora List) with most species ridged (from five to multiridged or multistriate) and some unridged. In the anterior nectophore, the hydroecium is typically very shallow, and the size, shape and position of the somatocyst, and the divided mouthplate are specifically diagnostic. Many species are rare to very rare, and their posterior nectophores and eudoxids unknown. A few have very small anterior nectophores (3–4 mm long), but most are intermediate (8–12 mm long), with some (*L. achilles*, *L. conoidea*, *L. fowleri*, *L. hardy*, *L. havock*, *L. hostile*, and *L. multicristata*) reaching from 15 to 25 mm in length. Forty-two percent of species inhabit epipelagic layers offshore, and several species make significant contributions to mid-water siphonophore assemblages (*L. conoidea*, *L. multicristata*, Mackie et al. 1987). A number of small fragile and rare multistriate species inhabit the deeper and calmer meso- and bathypelagic layers of temperate waters (*L. lelouveteau*, *L. quadriculata*, *L. grimaldii*, *L. exeter*).

Family Abylidae

This is another well-known and successful diphyomorph family of ten species, which are most abundant in tropical surface waters and differ from diphyids in having more prismatic and facetted nectophores, with serrated ridges and teeth and a posterior nectophore larger than the anterior one (Fig. 20e). This large nectophore provides the main propulsive force for abylid locomotion (Totton 1932) and also protects the contracted stem in a long hydroecium enclosed by a serrated longitudinal flap on the inner surface of the left hydroecial wing. Several aspects of the family

are summarized in Table 3. Abylids are a stable and long-known group, like the diphyids, with seven of the ten species already introduced by 1860 and the remaining three by 1925 (WoRMS Siphonophora List). Species diagnoses are based on the characters of the anterior nectophore, and are well summarized by Pugh (1999b), who also lists often used synonyms for five of the ten valid species.

Two subfamilies are recognized. The Abylinae include six species in two genera (*Abyla* and *Ceratocymba*), with a small facet at the anterior end of the anterior nectophore which leads the colony during locomotion. The Abylopsinae includes four species in three genera (*Abylopsis*, *Bassia*, and *Enneagonum*), with a small leading ridge at the anterior end of the anterior nectophore instead of a facet in *Abylopsis* and *Bassia* and a very differently shaped pyramidal anterior nectophore in *Enneagonum hyalinum*, which never develops a posterior nectophore.

Abyline species are all epipelagic and rare worldwide except for *Ceratocymba sagittata*, which is common and mainly epipelagic, with a slightly broader latitudinal range than other abylines, although this subfamily is absent from the Mediterranean. The anterior nectophore of most abylines is only 8–13 mm in length, but in *C. sagittata* it can reach 25 mm (Pugh 1999b). This species also has a pointed anterior extension beyond the small leading facet, and whole colonies of *C. sagittata* can reach 45 mm in length (Totton 1965, Fig. 140), which is very long for a diphyomorph species. *Abyla* species have anterior nectophores which are rectangular in lateral view, variable in width, and with a long hydroecium into which fits the prominent apophysis of the posterior nectophore. The latter are larger when mature in abylines, as noted above, and differ in width and number of teeth on the internal flap of the left hydroecial wing (Pugh 1999b). *Ceratocymba* comprises three species, which, in addition to *C. sagittata*, include the conspicuous but smaller opaque, rare, and sturdy species *C. dentata* and a third rare species *C. leuckarti*.

Abylopsines include three mainly epipelagic and common tropical and subtropical species: *Abylopsis eschscholtzii*, *A. tetragona*, and *Bassia bassensis*, which all occur worldwide and in the Mediterranean (Mapstone 2014 Table 2; Pugh 1999b). Anterior nectophores of *Abylopsis* and *Bassia* are similar, but can be distinguished by their somatocysts, which in *Abylopsis* terminate in a thin diverticulum. *A. tetragona* has a relatively longer posterior nectophore than *A. eschscholtzii*, although that shown in Fig. 20e has not yet reached its maximum length (see Totton 1965, Fig. 149, for a mature colony). These three species are often abundant in tropical siphonophore assemblages, together with certain tropical diphyid species (Lo et al. 2012), and *Bassia bassensis* is distributed throughout tropical and subtropical latitudes of all oceans and is also particularly tolerant of the varied environmental conditions found in neritic habitats (Lo et al. 2012); the ridges of this species are also tinged blue in life. *Enneagonum hyalinum*, in contrast, is a large and relatively uncommon abylopsine, from both tropical and temperate latitudes, with a pyramidal nectophore having nine points, a slim anteriorly directed somatocyst, and a relatively small nectosac. This shape is unwieldy, suggesting that this species is an ineffectual swimmer with a nectosac that can do little more than simply counteract the pull of gravity while floating in the water column (Totton 1932).

Conclusions and Future Directions

Forty three morphological siphonophore taxa have so far been successfully sampled for two genes, a comprehensive molecular consensus generated, and some interesting new characters and relationships revealed. More such characters and relationships might be found if further taxa are sampled (particularly for calycophorans and also for other physonects) and wider suite of genes investigated, although CO1 should be omitted, as this gene is unsuitable for detecting interspecific differences in Siphonophora. Investigation of mechanisms of tentillum discharge in different species and prey consumption in a wider range of taxa might also produce interesting new findings. Sampling of siphonophores and other zooplankton in areas not so far well studied worldwide (there are many) would also be informative, particularly if specimens of further species with gastrozooids containing prey items are collected, and might show further connections between siphonophores and other taxa in mid-water food webs, such as that recently discovered by Burford et al. (2014).

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Systematics and Evolution of the Conoidea 17

Nicolas Puillandre, Alexander E. Fedosov, and Yuri I. Kantor

Abstract

The highly diverse toxins of cone snails have been known since the 1970s; however, the evolutionary processes that led to both the species and toxin diversity in the group are only recently being explored. Furthermore, their closely related, also venomous but much more diversified, allies in the superfamily Conoidea remain largely unknown, with most species still undescribed and only few toxins characterized for a handful of conoideans other than cone snails. This chapter is a review of the literature dealing with systematics and evolution of the Conoidea. In particular, it will be shown how new hypotheses on the evolutionary processes have emerged from interdisciplinarity between ecology, taxonomy, phylogeny, anatomical study, and toxinology. It is becoming increasingly well documented that conoidean diversification is actually linked to toxin diversification: recent results tend to show that the venom apparatus played a major role in the evolution of the group by offering sets of unique molecular adaptations for efficient interactions with other taxa of marine animals. These, in turn, enhanced capacities of conoideans to efficiently compete for new ecological niches, a remarkable example of which is the appearance of fish hunting in cone snails. Speciation in conoideans was also promoted by other factors, e.g., episodic losses of planktotrophy that led to reduced dispersal abilities and intensive allopatric differentiation. Testing such hypotheses remains primarily based on the accumulation of data on the diversification patterns (in particular on

N. Puillandre (✉)
Institut de Systématique, Evolution, Biodiversité ISYEB – UMR7205 – CNRS, MNHN, UPMC, EPHE, Muséum National d'Histoire Naturelle, Sorbonne Universités, Paris, France
e-mail: nicolaspuillandre@gmail.com

A.E. Fedosov (✉) • Y.I. Kantor (✉)
A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia
e-mail: fedosovalexander@gmail.com; kantor.yuri1956@gmail.com

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_19

the systematics), and there is still a long road ahead to achieving a full understanding the evolutionary success of these remarkable mollusks.

Keywords

Conoidea • Taxonomy • phylogeny • Evolution • Conotoxins • Venom apparatus

Contents

Introduction

Although it does not come directly to mind when thinking of venomous organisms, the superfamily Conoidea challenges snakes, scorpions, and spiders for the title of most diverse taxon both in terms of species and toxins produced. Conoidea (Toxoglossa) constitutes a group of highly diversified marine gastropods, found in all oceans, from the tropics to the poles, and at all depths. It represents one of the most abundant groups of mollusks in bathyal and abyssal environments. Among them, cone snails (= Conidae) and the toxins they produce ("conotoxins") have been studied intensively since the 1970s. Focus has been on cone snails mainly because they are bigger, easier to sample, and overall better known than other conoideans, mainly thanks to the amateur shell-collecting community attracted by their shape and color variation and the fascination of deadly species. However, with around 850 species, cone snails only represent the tip of the iceberg: conoideans include 5,000 described species, but it is estimated that there may be 10–20,000 species in total. Research on Conoidea toxins are now expanding to other families, but cone snails remain more accessible, better known, and thus by far the most studied. Cone snails are well established as an important model in pharmacology; the numerous toxins they produce, mostly acting on ion channels, are characterized by a remarkable specificity to channel subtypes. Cone snails thus constitute a vast reservoir of natural peptides with multiple potential applications in both therapeutics and neuroscience.

Initially, studies on conoideans essentially followed two routes. Toxinology mainly focused on describing the molecular diversity of the toxins, guided by the motivation of understanding the functional mechanisms of envenomation and ultimately identifying potential therapeutic applications, while evolutionary biology relied on the accumulation of knowledge on the diversity of venomous organisms, including their toxins, to understand the evolutionary processes that lead to the apparition of such diversity. These two directions remained generally weakly correlated until recently, when a number of complex studies that bridged this gap through synthesizing of interdisciplinary data started to appear. It resulted in the realization that the diversification of the conoideans is better explained by integrating the processes occurring at the molecular level, and vice versa, and that taking into account phylogenetic, biological, and anatomical information enhances the understanding of the functional properties of the toxins. This chapter will thus be devoted to reviewing the knowledge of the biology, ecology, **systematics**, anatomy, and toxinology of the Conoidea, as well as the different evolutionary hypotheses proposed to explain this astonishing diversity of species and toxins.

First, the patterns of diversification related to (i) biology and ecology, (ii) systematics (**alpha-taxonomy** and phylogenetic relationships), (iii) venom apparatus, and (iv) toxin diversity will be discussed. As illustrated in Fig. 1, these different characters provide the arguments to propose hypotheses on the evolutionary history of the Conoidea and in particular on their diversification process. The last section will demonstrate how these patterns can be combined to understand how this group became one of the most successful taxa of marine animals.

Biology and Ecology

General Characteristics and Biology

Similar to most neogastropods, conoideans are carnivores, mostly, active predators. They are characterized by a venom apparatus (although secondarily lost in several lineages; see the section on "Venom Apparatus" below), associated with a highly modified radula used to inject venom in their prey, and to defeat competitors and/or predators (Dutertre et al. 2014a; Olivera et al. 2014). Several cases of human death caused by a contact with cone snails have been reported, with *Conus geographus* accounting for most of them (Dutertre et al. 2014b), but other species have also been involved in accidents (Haddad Junior et al. 2009).

Most species of conoideans, as well as the majority of other gastropods, are dextral, but some, including several species of *Antiplanes*, Pseudomelatomidae (Kantor and Sysoev 1991a), and one species of cone snail, *C. adversarius* (Hendricks 2005), possess a sinistral shell. Conoidea can be readily segregated into three groups (Fig. 2) by shell shape: (1) cone snails (**Conidae**) with conical shells and proportionally very low spire, high last adult whorl, and narrow slit-like aperture; (2) family **Terebridae** (or so-called Auger snails) with very tall multi-whorled spire, low aperture, and a very short siphonal canal; and (3) the rest of Conoidea that are

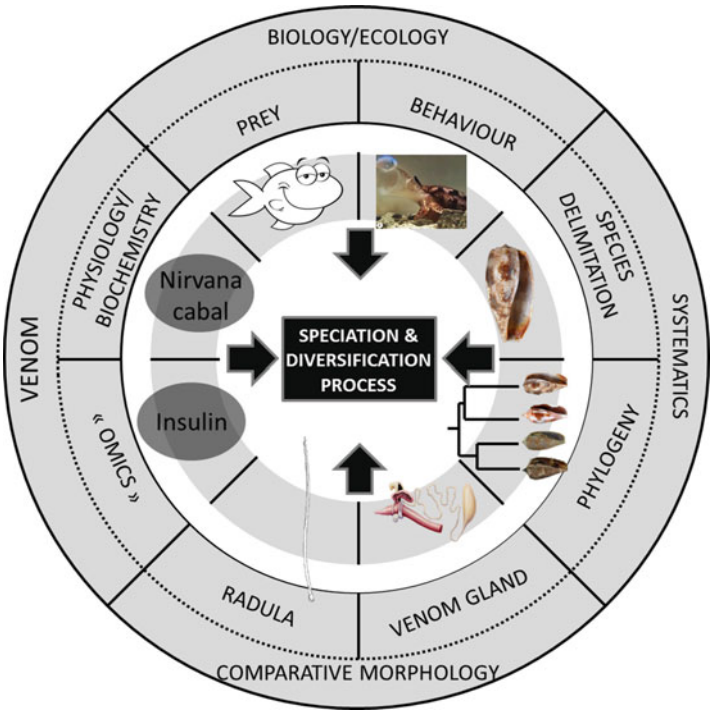

Fig. 1 Fields of research that were until recently quite independent now feed off each other to propose hypotheses on the speciation and diversification process. The outer ring lists the different fields and type of information for each field (non-exhaustive list); the inner ring provides an example for the species *Conus geographus*

characterized by intermediate shell proportions, and are collectively referred to as "**turrids**." While the two former groups are rather compact and easily recognizable and correspond to distinct phylogenetic clades, the "turrids" are extremely variable and comprise an array of lineages that do not form a natural grouping (the polyphyly of turrids has been convincingly demonstrated – see "Systematics" section below). Nevertheless, many "turrids" share one characteristic feature, a so-called anal sinus on the adapical part of the labrum (outer aperture lip), which allows their easy recognition. However, this feature is also present in some non-conoidean gastropod taxa (e.g., family Bursidae and subfamily Typhinae in the Muricidae) and at the same time is absent in many groups of "turrids."

Some lineages of Conoidea have a reduced or entirely lacking operculum. The protoconch can be multispiral or paucispiral, corresponding to planktotrophic or non-planktotrophic larvae, and reflecting high or low dispersal abilities, respectively (Jablonski and Lutz 1980). Likewise in other caenogastropods, sexes are separate and the fertilization is internal in all conoideans. One case of sexual dimorphism of the shell has been reported in *Gemmula* (Kantor and Sysoev 1991b). Egg capsule morphology is variable and well known in cone snails (Kohn 2012).

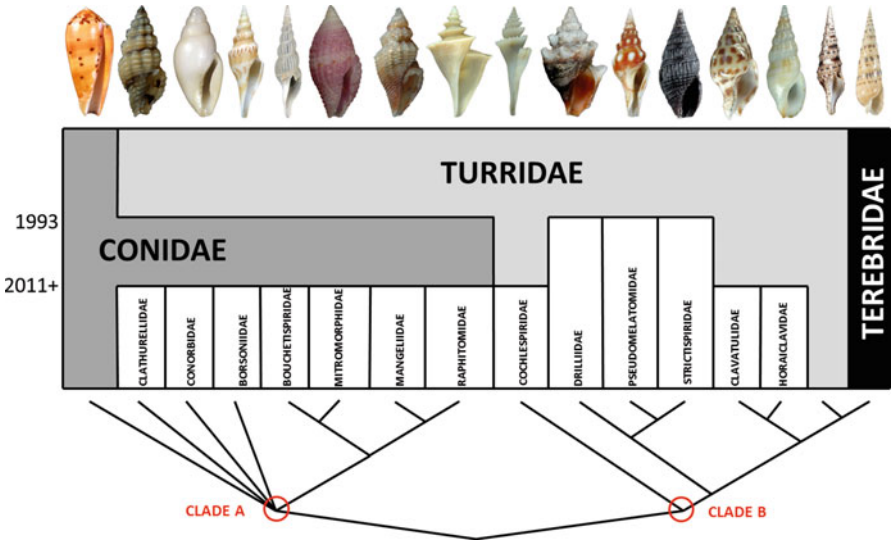

Fig. 2 Successive classification schemes proposed for the Conoidea. *Top row*, classification mainly based on shell characters (Powell 1966); *middle row*, classification based on anatomical and shell characters (Taylor et al. 1993); and *bottom row*, classification based on molecular data (Puillandre et al. 2011a; Kantor et al. 2012a), with the corresponding phylogenetic tree. The validity of the Strictispiridae remains to be tested. The Turridae experienced a drastic reduction of its included diversity; the Conidae have been successfully extended and then reduced to its original concept (but see Tucker and Tenorio (2009) for subdivisions within the Conidae); the Terebridae is the only family that has remained unchanged

Feeding Habits and Habitat

The unprecedentedly rich radiation of the Conoidea is usually attributed to a strict prey specialization, which is assumed to apply to most members of the superfamily (Taylor et al. 1980). Conoidean preys are mostly **polychaetes**, and less frequently nemerteans, hemichordates, and mollusks including gastropods (Miller and Morton 1990), bivalves (Fujikura et al. 2009), and even cephalopods (Marshall 1981). Cone snails are remarkable in this respect, as they are also able to feed on fish (Kohn 1956) and crustaceans (Biggs et al. 2010). Apart from the cone snails, preys are known only for around 50 species, with most data coming from the examination of the gut contents. Duda et al. (2009a) confirmed the diet of *Conus ebraeus* using a DNA barcoding approach. The type of prey in cone snails can also be inferred from indirect evidences – the morphology of the radula (Tucker and Tenorio 2009), or using "reverse ecology" (Olivera 2002), from the composition of the venom.

Feeding strategies observed on cone snails are diverse ("hook-and-line" = " taser-and-tether" = harpoon strategy, "net fishing," "strike-and-stalk" (Olivera et al. 2014, 2015)), even in fish-hunting species, to which most observations refer (Olivera et al. 2014). These different strategies are also associated with diverse behaviors as described for the fish-hunting cone snails, including mimetic with

brittle star arms and worms, or group attacks (Olivera et al. 2015). Scavenger behavior has also been reported in *Californiconus californicus*, the cone snail species with the widest prey spectrum, feeding on worms, mollusks, shrimps, and fish (Biggs et al. 2010). Remarkably, some cone snails are able to engulf a prey that is nearly equal to their own size, the ability facilitated by the reduction of the inner shell walls (Kantor 2007a). One specimen of cone snail typically feeds on a single prey per night (Kohn 1959), but see Olivera et al. (2015). Other specific behaviors likely related to feeding, including "surfboarding," have been described for Terebridae (Miller 1970).

Several conoidean species, even when phylogenetically closely related, can exist in sympatry, but probably by utilizing different niches (e.g., if the prey type is the same, the size of the prey will differ (Kohn 1980)). Up to 30 species of cone snails can be found in a single site, which was demonstrated for both recent (Muttenthaler et al. 2012) and fossil species (Hendricks 2015); some unpublished data suggest that up to 70 species can co-occur in a single locality (P. Bouchet pers. com). Typically, closely related *Conus* species will feed on different prey species (Kohn 1980; Duda et al. 2001). The same pattern was shown for terebrids: Fedosov et al. (2014) applied stable isotope analysis (SIA) to demonstrate existence of the resource partitioning in sympatric species of Terebridae that indirectly proves the alternate feeding specializations in multiple species of the family co-occurring within one habitat. In turn, fish (Simpson et al. 2013) and turtles (Behera et al. 2015) have been reported to feed on conoideans. In addition, the analysis by Dutertre et al. (2014a) implied that one of the important natural enemies of cone snails are octopi. Until recently, the use of the venom apparatus to defeat competitors or predators (and not to capture preys) was mostly hypothetical, relying mostly on observations in aquaria (Aman et al. 2015; Jin et al. 2015) (see "Speciation and Diversification" below).

Three species of cone snails are currently listed as critically endangered, 11 as endangered, 27 as vulnerable, and 26 as near-threatened in the IUCN Red List (Peters et al. 2013). Peters et al. (2015) also shown that 70 % of the red-listed species (regardless of status) are known from areas that are presently highly impacted by humans. In addition, over-sampling should not be neglected in considering threats to cone snails, especially by shell collectors (Duda et al. 2004).

Species Diversity

The enormous species diversity contained within Conoidea makes it one of the most speciose groups of marine animals: the World Register of Marine Species (WoRMS, http://www.marinespecies.org/, accessed in November 2015) lists 4,710 valid species for which 9,492 names are available. However, there are several lines of evidence suggesting that this number is, in fact, a great underestimation of actual conoidean diversity, and recent estimations (Bouchet et al. 2009) suggest that there are probably as many as 20,000 species of Conoidea. These estimates are based on the proportion of undescribed species calculated in well-sampled local faunas, in particular in New Caledonia (Bouchet et al. 2002, 2009). The high proportion of

undescribed species is, in part, a result of their rarity: 41 % of the turrid species in New Caledonia were represented by only a single specimen in the data set of Bouchet et al. (2009), and 73 % of the turrid species in New Caledonia were recovered only as dead shells (Bouchet et al. 2002). Remarkably, only 17 % of these species were also found in the surrounding archipelago. The other factor, which makes the adequate documentation of conoidean fauna rather hard, is a high proportion of very small species that are difficult to collect and study.

With 827 valid species, Conidae is currently considered the most diverse family; however, this is probably the family with the lowest proportion of undescribed species. If the proportion of undescribed species is taken into account, the families Pseudomelatomidae, Raphitomidae, and Mangeliidae may well be even more speciose. All these results tend to converge on the idea that a large part of Conoidea diversity remains unknown.

Like all shelled mollusks, conoideans have a large fossil record (Hendricks 2005; Hendricks 2015; Todd and Johnson 2013). The first fossil confidently identified as a cone snail is dated to 50–55 MY, while the oldest confident record of turrid in the fossil records is dated to the Cretaceous (Powell 1966); however, the uncertainty in the attribution of some fossils to the Conoidea remains important, and the minimum age of the group remains to be defined.

Systematics

Phylogeny and Classification

Conoidea (or Toxoglossa) is included in the order Neogastropoda (Mollusca, Caenogastropoda) in a rank of superfamily, together with five other currently recognized superfamilies, Cancellarioidea, Buccinoidea, Muricoidea, Olivoidea, and Turbinelloidea (Fedosov et al. unpublished). Because of their distinctive shell shape, compared to the rest of the Conoidea, the cone snails (Conidae) and the auger snails (Terebridae) were established as separate families in the earliest classifications of the group (e.g., Fischer 1887) (Fig. 2). The major confusion lay with the remaining Conoidea, which was subject to numerous alterations. Sometimes, they were included in Conidae (Thiele 1929), but most often everything besides the cones and terebrids were placed into the very heterogeneous family Turridae (or Pleurotomidae). Hedley (1922) correctly remarked that the family Turridae "is more perplexing than any other molluscan family."

The principal scheme, with three families recognized within Conoidea, persisted until the 1990s with a few modifications concerning the subdivision of the Turridae made by some later authors (Kilburn 1983; McLean 1971; Morrison 1965); these modifications essentially multiplied the number of the subfamilies in the Turridae, but their relationships remained unrevised. A first revolution occurred in 1993, when Taylor et al. (1993) proposed a completely new classification based on the **cladistic** analysis of the combined data set of shell, radular and anatomical characters (earlier classifications were built upon the "intuitive" approach using mainly shell

characters). Six families were proposed within the Conoidea, including the family Conidae extended to include five subfamilies earlier placed in Turridae. The names Conidae, either restricted to the cone snails or including also several turrid groups, and Turridae, either encompassing all the conoideans except cone and auger snails or restricted to a single group of turrids, have been thus used to designate different taxonomic concepts in the different classifications. Tucker and Tenorio (2009) proposed another classification of the Conoidea, based on the previous ones but also on the published molecular phylogenies. They proposed to split the Conoidea in two superfamilies, Conoidea and Turroidea (corresponding to the clade A and B defined in the molecular **phylogeny** of Puillandre et al. (2008)), with a total of 15 families assigned to them, the traditional Conidae being split into four different families.

The large panel of alternative classifications proposed for Conoidea attests to the difficulty encountered by taxonomists. The differences between the classifications are, at least partly, the consequence of the use of different characters (shell, radula, anatomy, DNA) by the taxonomists; what is considered an essential character by one taxonomist can be regarded as nonsignificant by another. Different schemes of relationships resulting when different sets of characters are analyzed are mainly explained by the fact that morphological evolution of Conoidea presents multiple cases of **homoplasy** or retention of ancestral polymorphism. Thus, the phylogenetic signal of many characters is masked to the extent that they cannot be used to infer phylogenetic relationships. Whereas DNA characters are, by definition, genetically determined, phenotypic traits, such as shell, radula, or anatomical features, are not necessarily genetically determined and can vary with the environment even when the genome remains unchanged.

The radula, in particular, although in general useful to infer phylogenetic relationships (Tucker and Tenorio 2009; Kantor et al. 2008), is known to be shaped by the diet. As phylogenetically distant conoideans can feed on similar preys, they may acquire strikingly similar radulae (Olivera et al. 2015; Castelin et al. 2012), which therefore would not reflect phylogenetic relationships. The same conclusion has been reached for shell characters: molecular phylogenies revealed that highly similar shells can actually correspond to two different families (e.g., almost identical representatives of the former genus "*Leucosyrinx*" are now placed in the *Sibogasyrinx*, Cochlespiridae and *Leucosyrinx*, Pseudomelatomidae). Even more prominent are the cases where the counterparts not only belong to different families but to different major clades of the Conoidea, e.g., *Cochlespira* (Cochlespiridae) versus *Toxicochlespira* (Mangeliidae). Needless to say, misinterpretation of shell morphology has led to erroneous placements of many conoidean species and genera; these were resolved only when additional morphological or molecular data were involved. Once the evolutionary lability of the shell in Conoidea was demonstrated, other sources of phylogenetic information were examined. In particular, the high taxonomic value of the protoconch was consistently promoted by Powell (1966) and has been widely used in **taxonomy** (Bouchet and Waren 1980). Indeed, some high-level conoidean taxa demonstrate characteristic sculpture patterns of the multispiral **protoconch**. However, among the many types of sculpture found in conoideans, not

a single one is shared by all the members of a clade, and only a few of them are found in only one lineage. In addition, all species with non-planktotrophic development have a paucispiral protoconch, either weakly sculptured, or with no sculpture whatsoever, and their placement therefore was troublesome. Using morphological characters only, Bouchet (1990) discouraged the separation of species with identical teleoconch but different types of protoconch in different genera as was suggested by Powell (1964) and instead proposed that even sister species could have different types of protoconch morphology (planktotrophic vs. non-planktotrophic). Molecular analyses confirmed this hypothesis, the "planktotrophic larvae" character state being lost regularly during the evolution of the Conoidea (Cunha et al. 2005; Duda and Palumbi 1999; Fedosov and Puillandre 2012; Remigio and Duda 2008).

Several phylogenies have been proposed for the Conoidea that combine molecular data and cladistic methods; the latest of them (Puillandre et al. 2011a) provided a basis for the simultaneously published classification of the superfamily (Bouchet et al. 2011). Fifteen families were recognized in this classification within the superfamily Conoidea, with the family Conidae including all cone snails. One year later, the sixteenth family, Bouchetispiridae, was added (Kantor et al. 2012a). Although some previous classifications, not based on molecular data, contradicted each other and are not in full agreement with the molecular-based classification, some phylogenetic relationships were actually already proposed in the literature, and in particular several features of the Taylor et al.'s classification (Taylor et al. 1993) were actually confirmed with DNA characters. It should also be noted that the classification by Bouchet et al. (2011), now widely accepted, is based on the molecular phylogeny and thus does not include fossil lineages, contrary to the shell-based classifications. Hendricks (Hendricks 2005) published the only phylogeny of Conoidea (more specifically, cone snails) that includes both recent and fossil taxa, combining morphological and molecular characters. Smith and Hendricks (Smith and Hendricks 2013) explored the possibility of including "cladistic pseudofossils" (recent species for which the molecular information is discarded) in a phylogeny based on both morphological and molecular characters, to test whether morphological data alone can place the species in the same position as the molecular data: the success rate was, however, below 50 %.

Molecular phylogenies restricted to subgroups within the Conoidea have been published in abundance. They are especially numerous for cone snails (listed in Puillandre et al. (2014a)) and scarcer for Terebridae (Castelin et al. 2012; Holford et al. 2009a, b) or Turridae sensu stricto (Fedosov et al. 2011; Heralde et al. 2007, 2010; Puillandre et al. 2012a). Other phylogenies that have been published are either limited to a few species only or reproduce already published phylogenies. Finally, species delimitation articles (see the "Alpha-taxonomy" section) often include a molecular phylogeny, but their scope is restricted.

Genome-based approaches have also been used in Conoidea: Bandyopadhyay et al. (2006) published the first **mitochondrial genome** of any Conoidea, *Lophiotoma cerithiformis* (Turridae), and several others have been published since then (*Conus textile*, *Conus consors*, *Conus borgesi*, *Oxymeris dimidiata*, *Fusiturris similis*, *Conus tulipa*, and *Conus tribblei*). Hu et al. (2011) published a preliminary

version of the nuclear genome of *Conus bullatus,* and Barghi et al. (2015a) published the genome of *Conus tribblei*. Additionally, a karyological analysis of *Conus magus* revealed a diploid chromosome number of 32 (Dalet et al. 2015). Several transcriptomes of venom glands of Conoidea are also available (Barghi et al. 2015b; Gonzales and Saloma 2014; Gorson et al. 2015; Prashanth et al. 2014; Robinson et al. 2014). Even though they have not yet been used to discuss phylogenetic relationships within the Conoidea, these data represent an interesting source of new molecular markers that could be used to improve the knowledge on the systematics of the Conoidea.

Alpha-taxonomy

Similar to classification, **species delimitation** in Conoidea is also a difficult task, for various reasons. First, similar to most mollusks, species delimitation and descriptions are mainly based on shell characters, even in recent years. Diagnoses, especially in older literature, are sometimes short, and applicable to several different species, leading sometimes to endless nomenclatural debates (Janssen et al. 2014). The high number of synonyms adds to the confusion. Second, it is generally difficult to identify and formalize discrete morphological characters. The shell variability often constitutes a continuum, and it can be very difficult to identify the limits between the species along this continuum. Species delimitations are thus often opinions based on an interpretation of the shell, and the absence of a clear description and formalization of the characters prevents any formal test of species hypotheses. Third, Conoidea diversity remains largely unknown, and each new expedition in the field collects new species to be described, but also new forms and new occurrences for already described species that requires taxonomists to modify their previously proposed species hypotheses. Finally, difference in morphological characters can reflect environmental differences (including, for example, radula convergence linked to prey preferences as described above) and not evolutionary relationships and species limits. These difficulties are not new: in 1884, Tryon stated "In no other group of mollusks is it so difficult to make a satisfactory classification as in the Pleurotomidae (= Turridae). The forms are exceedingly numerous, and known in many species to be very variable in their characters, whilst the material for the recognition of most of those described is generally scanty" (Tryon 1884).

As in other taxa, species delimitation tends to be more **integrative**, with joint analysis of morphological, molecular, and ecological characters. Other methods and characters have recently appeared in the toolbox, with geometric morphometry (Smith and Hendricks 2013; Puillandre et al. 2009) and molecular analyses (Cunha et al. 2008; Puillandre et al. 2011b), including conotoxin-coding genes (Duda et al. 2008; Puillandre et al. 2014b). The general tendency is toward splitting of previously recognized morphospecies in several species (e.g., Puillandre et al. (2010a)), subtle differences in the shell being found to be correlated with fixed genetic differences. Some exceptions can be found within cone snails, where geographic varieties have been described as different species in some cases.

Foregut Anatomy and Venom Apparatus

The anatomy of the foregut of Conoidea is quite variable, although several characters can be considered to be synapomorphies. The key apomorphy of the group is the **venom apparatus**, consisting of normally long convoluted tubular venom gland (often incorrectly referred to as "duct") and terminating in uni- or multilayered muscular bulb, serving as a propulsory organ (Fig. 3a, c). The venom gland is presently considered homologous to midgut gland of other neogastropods, while, based on the embryonic development of *Conus lividus*, the muscular bulb is a *de novo* structure (Page 2012). It is the sophisticated mechanism of venom delivery provided by radular functioning in conjunction with the venom gland, rather than the

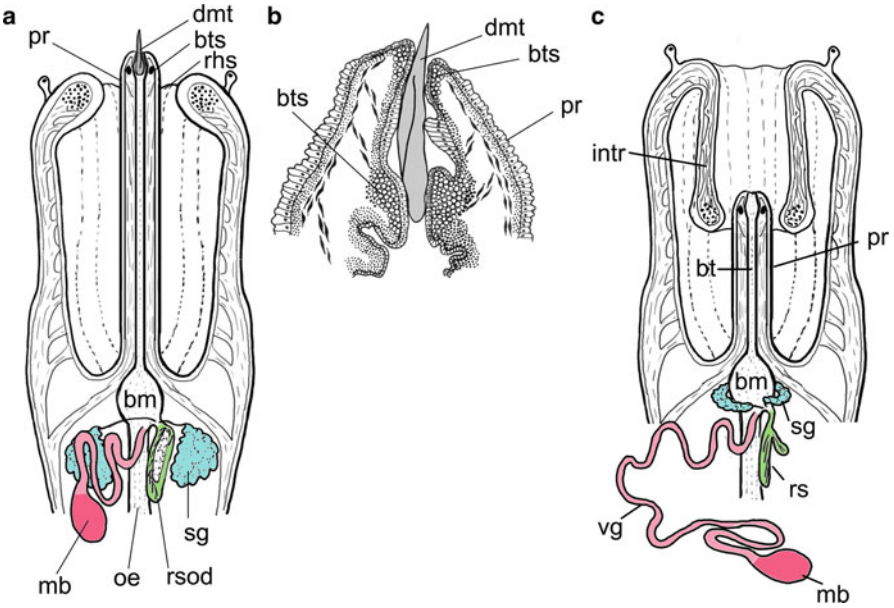

Fig. 3 Diagrammatic sections through the anterior foregut of Conoidea. (**a**) Anterior foregut of the Conoidea with non-hypodermic marginal radular teeth and odontophore (generalized representative of the clade B). A duplex marginal tooth detached from the sub-radular membrane is used at the proboscis tip for stabbing and envenomating the prey. (**b**) Section of the tip of the proboscis with the duplex marginal tooth held by sphincters of the buccal tube (actual specimen of *Aforia kupriyanovi* Sysoev and Kantor 1988, Cochlespiridae). (**c**) Anterior foregut of the Terebridae with developed rhynchostomal introvert, radula of hypodermic marginal teeth and lacking odontophore. A hypodermic marginal tooth detached from the sub-radular membrane is used at the proboscis tip. Abbreviations: *bm* buccal mass with radular apparatus at proboscis base; *bt* buccal tube leading from buccal mass to mouth on proboscis tip; *bts* buccal tube sphincter holding the base of the tooth at proboscis tip; *dmt* duplex (non-hypodermic) marginal tooth at the proboscis tip; *intr* rhynchostomal introvert, or pseudoproboscis; *mb* muscular bulb of the venom gland; *oe* esophagus; *pr* proboscis; *rhs* rhynchostome, or false mouth, through which the proboscis is everted; *rs* radular sac without odontophore; *rsod* radular sac with odontophore; *sg* salivary gland; *vg* venom gland

presence of the gland itself, that is the basis of the unique feeding mechanism of Conoidea (Kantor and Puillandre 2012).

In gastropods, the radular apparatus consists of a radular ribbon with numerous transverse rows of teeth (radula *per se*) and odontophore. The latter is a massive organ, consisting of several sub-radular cartilages and muscles, which provides the movement. The radular apparatus is situated in the buccal cavity in close proximity to the mouth and can be partially everted through the mouth opening. The radula serves as an integrated organ for rasping or gripping food objects. In the vast majority of Conoidea, the buccal cavity, together with the radular apparatus, is situated at the proboscis base (Fig. 3a, c). Consequently, the radula cannot be protruded through the mouth to directly contact prey. Instead, in the early stages of conoidean evolution, a peculiar and unique mechanism appeared, whereby individual marginal radular teeth are dislodged from the membrane, transported to proboscis tip, where they are fixed by a sphincter, and used for penetration of the prey's integument (Fig. 3b). The function of the radular apparatus as integral organ in the buccal cavity is thus very limited, since the prey is already captured and passed along proboscis before the contact with radula is made. Nevertheless, some functions may still be present, since in significant number of conoideans (clade B as defined in the molecular phylogeny of Puillandre et al. (2008)) the odontophore with muscles that provide movement of the radular membrane persists. The transformation of the radular apparatus in representatives of clade A was much more radical, and involved the complete disappearance of the odontophore, while the radular membrane is vestigial or even absent in some *Conus* (e.g., in *C. striatus*).

The **radula** itself is rather variable in Conoidea, having from five teeth in a transverse row (Drilliidae) to only two marginals (Powell 1966; Taylor et al. 1993; Kantor and Puillandre 2012). Due to a particularly important role in feeding, marginal teeth are probably under evolutionary selection, and their morphology reflects feeding specializations. In practically all groups of Conoidea of clade B, the marginal teeth are reinforced by different elements, and they have accessory limb in "duplex" teeth, or thickened edges, or obtain semi-enrolled form (trough shaped) and rarely even become hypodermic (Kantor and Taylor 2000) (Fig. 4d, g, h). The use of separate marginal teeth at the proboscis tip was demonstrated initially in clade B conoideans by serial histological sections of the tooth gripped by special sphincter (Kantor and Taylor 1991; Sysoev and Kantor 1987). Moreover, the teeth were gripped by the proboscis tip sphincter in a large proportion of studied preserved specimens. Since the studied specimens were not specially collected or treated, this is probably an indication that the tooth is permanently or most of time "primed" for use.

In Conoidea of clade A (which are better known since they include Conidae s.s.), the marginal radular teeth normally possess very characteristic (hypodermic) morphology, i.e., a hollow **harpoon** of syringe needle shape with holes at the base and tip (Fig. 4a–c). In such teeth, toxins are injected through the central tooth cavity. This, at the first glance, seems to be more efficient and may explain the broader known diet range (including fish) compared to clade B. According to phylogenetic analysis, the hypodermic teeth in clade A appeared only once. Another proof of

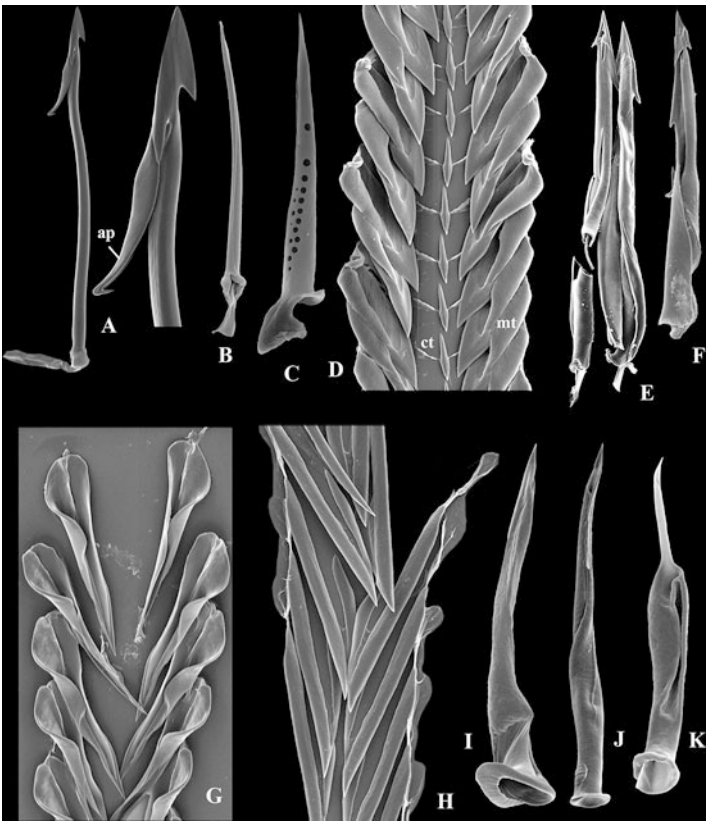

Fig. 4 Radulae of Conoidea. (**a**–**c**) Hypodermic marginal teeth of clade A of Conoidea, which includes only the species with hypodermic teeth and without odontophore. (**a**) Strongly barbed tooth of fish-hunting *Conus striatus* (family Conidae), and enlarged tip of the tooth. (**b**) *Bathytoma neocaledonica* (family Borsoniidae). (**c**) *Mangelia fieldeni* (family Mangeliidae). (**d**–**k**) Radulae of clade B of Conoidea, which includes species with odontophore. (**d**) Typical radula with duplex marginal teeth and central tooth, *Turridrupa jubata* (Turridae). (**e**) Semi-enrolled, nearly hypodermic marginal teeth, *Toxiclionella tumida* (family Clavatulidae). (**f**) Hypodermic marginal tooth, *Cruziturricula arcuata* (family Drilliidae). (**g**) Radula with semi-enrolled, trough-shaped marginal teeth, *Ptychobela suturalis* (family Pseudomelatomidae). (**h**–**k**) Different radulae of Terebridae. (**h**) Radula with duplex marginal teeth, *Clathroterebra poppei*. Next three figures depict hypodermic marginal teeth in Terebridae, originated independently in three clades, identified by molecular phylogeny: (**i**) *Terebra cingulifera*, (**j**) *Hastula lanceata*, and (**k**) *Myurella kilburni*. Abbreviations: *ap* accessory process, *ct* central tooth, *mt* marginal tooth

higher efficiency may be the independent origin of similar hypodermic teeth in unrelated lineages of Conoidea in clade B – in Clavatulidae (*Toxiclionella* (Kilburn 1985, Kantor and Taylor 2000); Fig. 4e), in Drilliidae (*Imaclava* (Shimek and Kohn 1981)), and at least three times in Terebridae (Castelin et al. 2012) (Fig. 4i–k). One of the still unexplained peculiarities of some hypodermic teeth that appeared in at least two non-related lineages (*Mangelia* from Mangeliidae of clade A, and *Impages* from

Terebridae of clade B) is the presence of numerous lateral holes in the tooth (Fig. 4c) (Imperial et al. 2007a). The morphology of hypodermic teeth in at least Conidae s.s. reflects their diet (Kohn et al. 1999), and it can change in ontogeny, probably due to diet shift (Nybakken 1990). Even within the fish-hunting cones, the radular morphology differs in species with different hunting strategy. Species that are known to use the "taser-and-tether" strategy have radular teeth that usually have a long accessory process (long outgrowth directed toward the tooth base; Fig 4a), often bearing an additional barb that can tether fish securely after a successful strike. In species using the "net" strategy, the teeth are poorly barbed at the tip (Tucker and Tenorio 2009; Olivera et al. 2015).

The unique **envenomation mechanism** of Conoidea is possible only by coordinated action of separate marginal teeth and proboscis. The proboscis grips the tooth, bringing it in contact with the prey and providing the impulse for injecting the tooth into the body of the prey. It also channels the flow of toxins from the venom gland. The mechanism of actual injection remains unclear, but for fish-hunting *Conus catus* the tooth is propelled by a high-speed ballistic mechanism after the proboscis tip makes contact with the fish skin (Schulz et al. 2004) and is then gripped by proboscis tip to retain control of the stung fish prey while retracting. Thus, the sphincter at the proboscis tip is able to maintain a strong grip on a relatively very thin object, only a fraction of a millimeter in diameter. Since the radula of Conoidea cannot be used for tearing or rasping prey (as it is either not an integral organ in clade A, or is situated at the base of proboscis and cannot protrude through mouth in clade B), the immobilized prey has to be swallowed whole. Thus, the very same sphincter of the mouth has the ability to undergo great expansion. For mollusk-hunting *Conus textile,* expansion of up to 50 times resting size was demonstrated (Kantor 2007b). The swallowing of particularly large prey relative to the size of the predator, such as mollusks or fish, is facilitated by a peculiar contraction of the proboscis. In *Conus,* many Raphitomidae, and some Mangeliidae, this forms multiple telescopic folds, and when in a contracted position, the proboscis occupies a small posterior part of the rhynchocoel (proboscis sheath) (Kantor and Taylor 2002).

In addition to the venom gland, there are salivary glands and in some groups, accessory salivary glands associated with anterior foregut. Their secretion remains grossly unstudied and different functions were proposed for salivary glands including cleaning the cellular debris in the radular tooth or "activating the venom" (Shimek 1975). Biggs et al. (2008) revealed transcripts whose predicted gene products, after posttranslational processing, strikingly resemble mature conopeptides belonging to the A-conotoxin superfamily.

It is worth mentioning the tendency in Conoidea toward reduction and complete loss of different foregut structures. First, radula-less turrids without proboscis, venom, or salivary glands were found in Raphitomidae (Smith 1967); later, this phenomenon was also recorded in several families of Conoidea other (Raphitomidae (Kantor and Taylor 2002; Kantor and Sysoev 1989); Horaiclavidae (Fedosov and Kantor 2008); Borsoniidae (Medinskaya and Sysoev 2003)). The loss of the venom gland is usually (although not always) accompanied by the loss of the radula and proboscis. A remarkable exception is the genus *Strictispira* (Strictispiridae), which

possess proboscis and large radulae with odontophore, but have no traces of the venom gland (Kantor and Taylor 1994). Multiple independent losses of different structures of the foregut were demonstrated for Terebridae (Castelin et al. 2012). Fedosov et al. (2014) demonstrated that the isotopic trophic niches of the radula-less terebrids are not wider than in those species, possessing radulae, which implies that the loss of radula does not affect degree of feeding specialization.

In some Conoidea, the anterior pre-tentacular region of the head becomes greatly expanded and forms either a large funnel (called rostrum in *Conus*, also present in many Raphitomidae) or eversible tube (pseudoproboscis, or rhynchostomal introvert). The latter is found in some Mangeliidae, most Raphitomidae, and all Terebridae (Taylor et al. 1993; Kantor and Taylor 2002) (Fig. 3c). The rostrum and pseudoproboscis are actively utilized in prey capture, as was observed for terebrids (Miller 1975).

Toxin Diversity and Evolution

Reviews on the structural and functional diversity of the toxins produced by the Conoidea, and in particular by the cone snails, as well as their potential or confirmed therapeutic importance, are numerous, and this will not be the purpose here (for a detailed review see, e.g., Olivera et al. (2014); Prashanth et al. (2014)). After a short introduction on these topics, the evolutionary aspects related to toxin diversity will be detailed.

What Is a Conotoxin?

Physiologically active peptides produced in the venom gland show some structural peculiarities that, although not shared among all types of molecules, allow a general description of conoidean toxins.

1. They are commonly short (12–40 residues) molecules with a high proportion of cysteines, expressed in the venom gland of cone snails (typically genus *Conus*). Their secondary structure is stabilized by the disulfide cross-links and achieved through the specific folding patterns. The rather conservative arrangement of Cys residues in the conoidean toxin peptides (*Cys* framework) is the major determinant of their specific conformation. Many *Conus* venom peptides, however, are either long, or contain few or no cysteine residues; a more general term "conopeptides" therefore refers to the whole diversity of *Conus* venom peptides (Puillandre et al. 2012b). Conopeptides are characterized by a high proportion of posttranslationally modified residues. Of these, some identified modifications (hydroxyproline, *O*-glycosylated serine, or tryptophan) are widespread in the animal world, while others (6-bromtryptophan, γ-carboxyglutamate, and sulfotyrosine) are exclusive for Conoidea (Bandyopadhyay et al. 2002). The venom gland peptides in other conoidean lineages, i.e., Turridae (turripeptides),

Terebridae (teretoxins), and Pseudomelatomidae (crassipeptides), are also disulfide rich, but are generally longer and less posttranslationally modified than those in *Conus*.

2. All studied conoidean peptides are synthesized in a same manner: the translation of a messenger RNA produces a peptide precursor, which consists of three conservatively arranged functional blocks. An N-terminal signal region followed by a pro-peptide region (which may be absent in some cases), and a mature peptide region on the C-terminal end of the precursor (Olivera 2006). The precursor undergoes maturation with formation of a specific pattern of disulfide cross-links, posttranslational modification of specific amino-acid residues, and removal of the signal and pro-peptide regions.
3. One of the remarkable characteristics of conoidean venom peptides is a disparity between extremely structurally diverse mature toxin regions (except for the Cys framework) and highly conserved signal and pro-peptide regions. Signal regions are used in classification of venom peptides into gene superfamilies (Puillandre et al. 2012b), designated by uppercase Latin letters. The three most widespread are the A-, O-, and M-superfamilies. The conotoxin families (designated with lowercase Greek letters – α to ω) are determined by the molecular target.
4. Most conopeptides target voltage- and ligand-gated ion channels that mediate dissemination of nerve impulses and neuromuscular transmission. Among the best studied are the ω-conotoxins (targeting presynaptic Ca channels), μ- and δ-conotoxins (blockers of Na channels), and α-conotoxins (antagonists of the postsynaptic nicotinic receptors – nAChRc). Cysteine-poor venom peptides target a variety of physiological pathways, e.g., conopressin (vasopressin receptors), conantokin (inhibitor of NMDA receptors), contulakin G (agonist of neurotensin receptors), χ-conotoxin Mr5A (blocker of noradrenalin transporter NET). The most recent discovery is the specialized fish-type insulin produced in the venom gland of *C. geographus* (Safavi-Hemami et al. 2015).
5. An outstanding property of the conopeptides is their extreme target specificity. Venom of one *Conus* species often includes multiple conotoxins of one family, each of which is "responsible" for its own receptor's subtype. For example, among ω-conotoxins of *Conus magus*, MVIIA acts specifically on Cav2.2 and MVIIB on Cav2.1, and neither of them shows any affinity to the other's molecular target (Nielsen et al. 1999). Even higher diversity and specificity was demonstrated for the α-conotoxins reflecting structural diversity and complexity of nAChRc (Jacobsen et al. 1997).
6. In complex conoidean venoms, individual peptides act in concert to achieve a major physiological effect. The functional groups of the conoidean venom peptides that affect multiple molecular targets within one physiological circuit, and thus act synergistically, are known as "cabals." "Thus, the evolution of conoidean gastropods has produced what is in essence a highly sophisticated equivalent to combination drug therapy" (Olivera et al. 2014).
7. The conoidean venom gland is compartmentalized, with distal and proximal portions of the gland specialized to produce different venom components. It was demonstrated that venoms produced in distal and proximal portions of the

Conus venom gland differ in functionality and may be discriminatively used by the cone snail for predation or defense in response to different external stimuli ((Dutertre et al. 2014a); see below for more details).

Phylogeny of Conoidean Venom Peptides

The principles upon which the classification of conoidean toxins is based were reassessed using a phylogenetic approach (Puillandre et al. 2012b). The traditional definition of the gene superfamilies, based on the similarities in the signal sequences, was generally confirmed. However, the astonishing diversity of potentially new superfamilies (or at least new signal sequences) revealed by the numerous recent **transcriptomic** analyses tends to somewhat blur the limits between superfamilies, with the signal diversity resembling a continuum of variability rather than a partition of clearly different signal sequences. Until recently, cysteine-rich and cysteine-poor conotoxins were considered to be two independent groups of *Conus* venom peptides; however, this distinction was not supported by the phylogenetic analysis. Several groups of cysteine-poor conopeptides were shown to share similar signal sequences with some well-established gene superfamilies of cysteine-rich conopeptides; therefore, in terms of relatedness, different groups of cysteine-poor conopeptides are not closer to each other than they are to some cysteine-rich conotoxins, and vice versa (Puillandre et al. 2012b). Thus, the classifications based on the **Cys pattern** and the function are purely practical. A given Cys pattern or function can be found potentially in several venom peptide superfamilies, and a given superfamily may include several Cys patterns and/or functions (Prashanth et al. 2014). Phylogenetic approaches also suggested a complex evolutionary origin of the conoidean toxins, with different peptides found in single venom recruited from a diversity of peptide-coding genes, with various physiological functions (Casewell et al. 2013). This remarkable complexity of conoidean venoms is conspicuously illustrated by the conkunitzins, structurally divergent from "traditional" conotoxins and bearing Kunitz domains, which are conserved among many animal lineages, and by recently discovered *Conus* venom insulins.

Molecular Mechanisms of Conopeptide Diversification

Several hypotheses have been proposed to explain the mechanisms underlying diversification of conoidean toxins leading to the recruitment of novel venom peptides. These include active hypermutational mechanisms (Olivera et al. 2014; Espiritu et al. 2001), lack of a mismatch repair system in the mature toxin (Olivera et al. 1999), recombination (Olivera et al. 1999), and exon shuffling (Pi et al. 2006). These generate a pool of "trial" venom peptides that may contribute to the efficiency of predation or defense or lead to a shift in the prey specialization; in any of these cases, the gene undergoes **positive selection** (Casewell et al. 2013; Duda 2008; Puillandre et al. 2010b), especially in the case of highly expressed genes (Chang and Duda 2014). Additionally, expression of some toxin genes may be inactivated, and

the silenced genes retained in the genome. Toxins **gene silencing** and revival give rise to the so-called *lazarotoxins* (Conticello et al. 2001), peptide products of some ancestral toxin genes reemployed and intensively expressed by some derived taxon. The aforementioned genetic mechanisms generate species-specific sets of expressed venom toxin genes (estimated between 100 and 200 per species) in cone snails. At the peptide level, additional mechanisms including alternative cleaving, posttranslational modifications, and N- and C-terminal truncations further increase the diversity of toxins in the mature venom (Dutertre et al. 2013), even if most (but not all, including toxins confirmed by proteomic approaches) represent rare transcripts (Prashanth et al. 2014).

It is generally accepted that each cone snail species possesses its own arsenal of toxins (Olivera 2006), partly shaped by the distinctive species-specific gene expression profiles (Conticello et al. 2001). The crucial question is how the processes underlying the hyper-variability of venom peptides (outlined in the previous paragraph) are coordinated to shape individual peptide repertoires in the diverging species. A recent study on the conopeptide expression patterns in two closely related *Conus* species (Barghi et al. 2015c) shed some light on this matter. Worm-hunting species of the subgenus *Splinoconus*, *Conus tribblei*, and *Conus lenavati* demonstrated unusual diversity of gene superfamilies expressed in their venom glands – 39 and 40, respectively, with 100 and 132 unique venom peptides identified in these species through high-throughput sequencing. Sixty-seven pairs of orthologs were identified in these two species, and 21 of these pairs showed identical mature toxin regions. Interestingly, highly expressed toxin gene superfamilies tend to show highly correlated expression levels, although may differ in the number of unique peptides expressed in each species, whereas the moderately expressed gene superfamilies show higher between-species variation in expression. Finally, about 20 % of toxin gene superfamilies expressed by each species (eight in *C. lenavati* and seven in *C. tribblei*) were not detected in the other. Thus, fine-tuning of the expression pattern and concomitant recruitment (revival) of the novel (lazaro-) toxins underlie divergence of the *Conus* venoms ((Barghi et al. 2015c); see also Chang and Duda (2014)).

Duda and Palumbi (2004) found identical toxins in the two clades of fish-hunting cone snails, which are supposedly not sister groups (although this remains to be demonstrated; see "Species and Diversification" section), which could be explained either by a convergence between the two clades, or by gene silencing. In the latter case, some ancestral toxins become increasingly highly expressed in distantly related clades that developed piscivory, while remaining silent in the non-fish-hunting clades. Alternatively, these toxins might also be expressed in worm-hunting cone snails, to be used not in predation but in defense (Dutertre et al. 2014a). Conversely, some remarkable cases are known (Imperial et al. 2007b) when two unrelated lineages of fish-hunting cone snails employ structurally highly divergent toxins, that specifically interact with same molecular target in the body of prey fish.

It should be noted that the complement of toxins varies among individuals of the same species; intraspecific and intraindividual variations of toxin diversity, either at the genetic level or when looking at the expressed toxins, have been reported in many studies (Prashanth et al. 2014; Jakubowski et al. 2005). Differences in toxin

composition and/or expression between different development stages in one species have also been documented (Chang and Duda 2016; Safavi-Hemami et al. 2011). Interestingly, these differences likely parallel the ontogenetic changes in radular morphology supposedly reflecting prey preferences that are different in juvenile and adult cone snails (Nybakken and Perron 1988). To add an additional degree of complexity, some studies have proposed that toxins can be produced in organs other than the venom gland ((Biggs et al. 2008; Jakubowski et al. 2005); but see Dutertre et al. (2014a)).

State of the Art of the Conoidean Toxin Diversity

Knowledge about toxins from Conoidea remained limited to cone snails until recently. A quick bibliographic search in the Web of Science (Fig. 5) suggests that within Conoidea, there is a clear bias in the effort of the toxinologist community toward cone snails; within cone snails, there is a bias toward *Conus*; and within *Conus*, there is a bias toward non-worm-hunting species.

Conoidean toxins are likely the most diverse, both in terms of numbers (estimated from the conoidean species diversity and the diversity of toxins in individual venom)

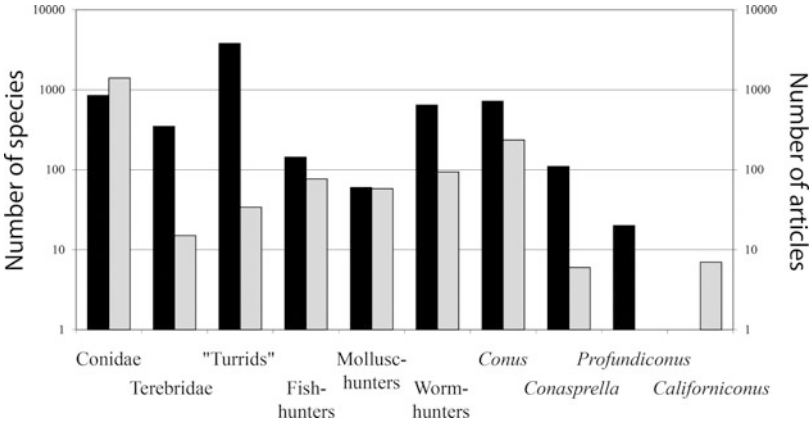

Fig. 5 Results of the bibliographic search, with the number of species (in *black*) and the number of published articles (in *gray*) for different groups of Conoidea and cone snails. Search was performed in the Web of Science with the keywords "Terebrid*" AND "*toxin*" in TOPIC, "Conus" AND "*toxin*" in TOPIC and "Turri*" AND "*toxin*" in TOPIC returned 15 articles refer to the Terebridae, 1405 to cone snails, and 34 to the "turrids," although they respectively represent c.a. 9 %, 16 %, and 75 % of conoidean diversity (Fig. 5). Among 1405 articles dealing with the cone snails, 236 cited a *Conus* species in the title. Of these, 77 articles were citing a fish-hunting species, 58 a mollusk-hunting species, and 94 a worm-hunting species; in total 68 different *Conus* species were cited. Although fish-hunting and mollusk-hunting species represent 17 % and 7 % of the total number of cone snail species, they are cited in 33 % and 25 % of the articles, respectively. The genus *Conasprella* represents 13 % of cone snail species but is cited in only six articles of the 236, while species of *Profundiconus* are never cited, and *Californiconus* is an exception, with seven citations for a single species

and molecular targets, compared to toxins in other groups of venomous organisms (Prashanth et al. 2014). Within *Conus* (sensu (Puillandre et al. 2015)), at least one toxin has been sequenced and eventually functionally characterized for fewer than 100 species (www.conoserver.org). A few species in other clades of cone snails (*Conasprella*, *Californiconus*) have been studied, with generally high proportion of new toxins that are not found in *Conus* (see Puillandre et al. 2012b; Kaas et al. 2012). In particular, half of the toxin superfamilies found in *C. californiconus* have never been found in *Conus* (Biggs et al. 2010). In the last 10 years, toxins from other Conoidea have been sequenced, although well-documented toxins remain very limited in number compared to the toxins of cone snails. At present, some data on the venom gland peptides exist for eight species in Turridae (*Polystira albida*, *Gemmula periscelida*, *G. speciosa*, *G. sogodensis*, *G. diomedea*, *G. kieneri*, *Lophiotoma olangoensis*, and *Unedogemmula bisaya*), six in Terebridae (*Hastula hectica*, *Terebra subulata*, *T. argus*, *T. consorbina*, *T. variegata*, and *Oxymeris maculata*), and one each in Pseudomelatomidae (*Crassispira cerithina*), Clathurellidae ("*Clathurella*" *cincta*), and Clavatulidae (*Turricula javana*) (Gorson et al. 2015; Imperial et al. 2007a; Aguilar et al. 2009; Heralde et al. 2008). Most toxin superfamilies are found in only one family of Conoidea, except one shared by Turridae and Conidae (Olivera et al. 2014), and another shared by a turrid *Lophiotoma olangoensis*, and a terebrid *Hastula hectica* (Imperial et al. 2007a; Watkins et al. 2006): the latter two groups are supposedly sister clades (Puillandre et al. 2011a). The venom peptides in *Hastula hectica* show extremely low frequency of posttranslational modifications, comparing to the conotoxins (Prashanth et al. 2014; Imperial et al. 2007a; Dutertre et al. 2010; Jakubowski et al. 2006) and the turritoxins (Olivera et al. 2014). Outside the Conoidea, peptides with structures and/or sequences similar to conotoxins have been found in mussels (Gerdol et al. 2015), whereas the toxins isolated from the polychaete genus *Glycera* are similar in many features to the turrid venom peptides (von Reumont et al. 2014).

Generally, toxin discovery has moved from a cDNA-based sequencing of more or less randomly selected taxa before 2000, to a **concerted discovery** approach (using systematics and transcriptomics to identify new lineages and new toxins) post-2000 (Olivera 2006; Fry et al. 2015; Puillandre and Holford 2010) and, more recently, to **venomics**, which combines proteomics and **next generation sequencing** (NGS)-based approaches to characterize transcriptomes (Prashanth et al. 2014; Kaas and Craik 2015). Still, all available data on conoidean toxin diversity and evolution are nuanced by the fact that toxin sampling of a venom gland is never exhaustive (Duda and Remigio 2008), although this bias is notably less (but not fully resolved) when using contemporary NGS-based approaches.

Speciation and Diversification

The knowledge reviewed in this chapter has been obtained by different research teams working in different scientific fields: e.g., systematics and morpho-anatomical descriptions were provided by zoologists; characterization of the toxins and their

molecular targets, as well as the physiological effects induced, by toxinologists. These fields of research remained generally disconnected from each other, with some notable exceptions, until the late 1990s, when more integrated approaches started to emerge. This synthesis will be illustrated with several examples taken from the literature, from the population level to macroevolutionary processes. Although the analytical methods and the concepts are not necessarily the same depending on the taxonomic level considered, the evolutionary hypotheses proposed to explain the observed pattern always rely on the integrated analyses of various type of data (e.g., DNA, morphology, distribution, ecology, toxins) (Fig. 1).

Given that one of the most striking features of the Conoidea is their extreme diversity, it is not surprising that one of the first evolutionary questions to be tackled is why there are so many species in this group. If the **allopatric** model, i.e., in which new species arise when populations become geographically disconnected, is the canonical **speciation** model for terrestrial organisms, the apparent continuity of the marine ecosystems makes this model less likely. Speciation for marine organisms is thus generally linked either to limited dispersal abilities and/or to habitat shift that would lead to reproductive barriers not necessarily linked to geographical isolation. Both these hypotheses have been tested, at least partly, in the Conoidea, and in particular, in cone snails. In most cases, these studies include the analysis of molecular data, either to reconstruct the relationships between the studied species and their close relatives, or to analyze the genetic differentiation between populations within a species.

Because the dispersal ability of gastropods can be deduced from the larval shell, several authors have tested the correlation between protoconch type and speciation rate. This hypothesis is generally tested using dated molecular phylogenies, calibrated using either fossils (in particular, the oldest cone snails or the divergence between *C. quercinus* and *C. lividus*) (Cunha et al. 2005; Duda and Kohn 2005) or molecular substitution rates (Duda and Palumbi 1999). Cunha et al. (2005, 2008) showed that the diversification of one of the two Cape Verde clades of cone snails is linked to the loss of planktotrophy, limiting the dispersal abilities and favoring the genetic differentiation of geographic populations, and ultimately resulting in speciation. Similarly, Kohn (Kohn 2012) established a link between dispersal abilities, estimated from egg size, and area of distribution.; The correlation remained valid when the phylogenetic signal was taken into account (i.e., the species with large distribution areas and long dispersal phases are not phylogenetically more closely related to each other than to species with short dispersal phase and more narrow distribution). Duda and Palumbi (1999) showed that planktotrophy has been lost repeatedly during the evolution of cone snails and that species with limited dispersal abilities are more numerous. This result could suggest that the speciation rate is greater in species that lost planktotrophy, but it can also be explained by the fact that the reverse transition (from non-planktotrophy to planktotrophy) is never observed (or at least never convincingly demonstrated). Thus, an equal speciation rate in both development types would lead to the same pattern. Although not correlating their results with dispersal abilities, Duda and Kohn (2005) also interpreted their result in an allopatric context: they delimited two main lineages in cone snails that diverged

33 MYA, one seemingly limited to the Indo-Pacific (large major clade – LMC) and the other to the East Pacific and the Atlantic (small major clade – SMC). This scenario has been rejected by Puillandre et al. (2014a) as a larger data set tended to show that the distinction between the two clades is more related to the bathymetry than to the geography.

Population genetics also helped to test whether geographic distances are correlated with genetic differentiation (Duda et al. 2012; Duda and Lee 2009a). Some cone snails in the insular regions of the Indo-Pacific are slightly divergent from the rest of their distribution (such as *Conus miliaris* are slightly divergent in Easter Island and *C. sanguinolentus* in Hawaii), whereas others in contrast show an absence of genetic structure in the whole Indo-Pacific (e.g., *C. chaldaeus*). Thus, high dispersal rates tend to homogenize the species with large distribution areas, this pattern being disturbed by the stochasticity of long-distance dispersal for the peripheral populations. Even if a species is characterized by highly dispersing larvae, some isolated archipelagoes may be difficult to reach, thus reducing the migration rate between the isolated populations and the others and increasing the genetic differentiation between them.

When the signal of allopatric differentiation, and thus speciation, is less clear, **ecological speciation** has been evoked to explain current distribution areas (Vallejo 2005). Numerous species of ecologically similar and phylogenetically closely related (at least for some of them) Terebridae have been found to co-occur on the same beach in Vietnam (Kantor et al. 2012b). An efficient pattern of resource partitioning among these species has been demonstrated, with the most abundant or most closely related syntopic species showing minimal overlap in trophic niches as indicated by stable isotope analysis (SIA) (Fedosov et al. 2014). Duda and Rolàn (2005), in a study similar to Cunha et al. (2005), showed that the higher number of species in the oldest island of the Cape Verde archipelago is not due to a longer diversification time but to a highest variability of habitats. Cunha et al. (2014) confirmed this hypothesis by comparing the high diversity of the cone snails in the Cape Verde Islands with their low diversity in the Canary Islands, proposing again that this difference is not linked to different biogeographical histories, but rather to ecological parameters such as a lower variability of habitats in the Canary Islands. More generally, and as for other venomous organisms, a link between toxin diversity, **prey shifts,** and speciation has been proposed as a major hypothesis to explain the evolutionary success of the Conoidea (Olivera 2006; Duda 2008). *Conus conco*, limited to the Marquesas Islands, was probably a peripheral population of *C. lividus*, a species present in the whole Indo-Pacific except in the Marquesas Islands. Both species having evolved different toxins, and prey shift may also be involved in this apparent case of peripatric speciation (Puillandre et al. 2014b). Similarly, Duda and Lee (2009b) proposed the hypothesis of "ecological release" for *Conus miliaris* in the Easter Island, where the species has supposedly less competitors and more diversified prey than in the rest of its distribution range. Furthermore, the genetic diversity of two toxin-coding genes is higher on this island, a pattern interpreted as resulting from a higher selection pressure. In this case, both geographic isolation and different environmental conditions would explain the diversification within a

species. Similarly, for *Conus ebraeus*, toxin-coding genes exhibit genetic differentiation between Okinawa, Guam, and Hawaii, although the COI gene does not (Duda et al. 2009a); the prey, identified using a metabarcoding approach, are indeed different in Hawaii. *Conus ebraeus* and *C. judeus*, two sympatric closely related species (but not sister species) and genetically clearly different, are actually characterized by different radulae and prey (Duda et al. 2009b), suggesting again that species occupy different ecological niches. Several authors (Barghi et al. 2015c; Chang et al. 2015; Phuong et al. 2015) also concluded that the dietary breadth influences the diversity in at least some toxin genes more than the type of prey, and Chang and Duda (2016) showed that the diet and the expression pattern of toxin genes can change throughout the life history of cone snail.

At a higher taxonomic level, Williams and Duda (2008) detected an increase in speciation rate in *Conus* sensu (Puillandre et al. 2015) around 20-25 MYA, a pattern found also in two other molluscan groups (*Turbo* and *Echinolittorina*). However, their study includes only 100 species, and even if they correct their results by considering that cone snails include 500 species, it still remains an underestimation of the total number of species. **Speciation rates** have also been estimated using the fossil record, and not calculated using time-calibrated phylogenies. Kohn (1990) described the fossil records of cone snails, with successive phases of expansion and reduction of the diversity, with a global tendency toward a less elongated shell, which could be linked to the radiation of the cone snails. More recently, Todd and Johnson (Todd and Johnson 2013) estimated the speciation rate in *Polystira* (Turridae) between 0.2 and 0.5 species per MY, thus identical to or even higher than the speciation rate in cone snails (Cunha et al. 2005; Kohn 1990). The absence of apparent variation across time in species richness for *Polystira* could be more linked to intrinsic (e.g., speciation favored by prey shifts and toxin diversification) than to extrinsic factors (e.g., geological events).

However, one of the most studied evolutionary patterns is the transition from worm-hunting cone snails (WHC) to fish-hunting cone snails (FHC) (and, to a lesser extent, to mollusk-hunting cone snails – MHC). Originally, two hypotheses, resulting from both phylogenetic and toxinological analyses, were proposed to support the idea that FHC and MHC arose from a WHC ancestor. More recently, other data have been added, perfectly illustrating the integrative approach (Fig. 1). Duda et al. (2001) first proposed that the transition was from WHC to FHC, and that this transition had occurred twice independently during the evolution of cone snails (giving birth to two lineages of FHC: the clade including the subgenera *Asprella*, *Afonsoconus*, *Textilia*, *Pionoconus*, *Embrikena*, *Gastridium*, and *Phasmoconus* on one hand and *Chelyconus* on the other hand). Subsequent molecular phylogenies also supported this result (Puillandre et al. 2014a; Espiritu et al. 2001), although it should be noted that the node on which this hypothesis is based has never been statistically supported. It should also be noted that not all the species in these subgenera, and even not all the subgenera, have been confirmed to be fish hunters by direct observations (Olivera et al. 2015); for several species, prey type has been deduced using a comparative approach, as illustrated in Fig. 6. More generally, Duda et al. (2001) also shown that the number of transitions from one feeding type to

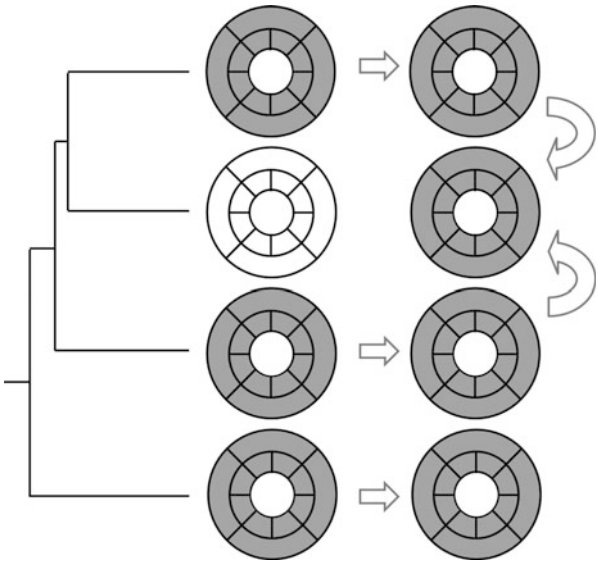

Fig. 6 Integration of different patterns. Available data on a given set of species (**a**, **c**, **d**) can be used to propose hypotheses on the evolutionary processes that led to the observed pattern. Furthermore, it can also be used to predict missing characters states, based on a given evolutionary hypothesis. Similarly, available data on some species (**a**, **c**, **d**) can be used to predict the character states for an unknown species (**b**), based on the phylogenetic relationships

another is limited in cone snails, a hypothesis supported also by the most recent molecular phylogeny (Puillandre et al. 2014a).

Toxins provide support for this hypothesis, but have also been used to propose more detailed scenarios of the transition from WHS to FHS and WHS species, independently from the phylogenetic analysis. In the lightning-strike cabal, some analogous toxins have been recruited independently in the two lineages of FHS (Olivera et al. 2014), while others were already present in the WHS ancestor, supporting the hypothesis that both FHS clades are independent, and that toxins acting on fish were already present in WHS (Imperial et al. 2007b). To explain this result, it was proposed that WHS were using toxins to deter competitors, such as fish (Aman et al. 2015; Imperial et al. 2007b). Alternatively, it has been suggested that WHS used these toxins to defend themselves from predators (i.e., fish), because these toxins were found in the proximal part of the venom gland, used for the defense (Dutertre et al. 2014a; Jin et al. 2015), although it remains to be shown that this pattern is common to all cone snails (but see Prashanth et al. (2016)). Furthermore, in FHS, the toxins found in the attack venom do not include the toxins supposed to be included in the motor cabal (see ► Chap. 6, "Revising the Role of Defense and Predation in Cone Snail Venom Evolution" by Lewis et al.).

Conclusion and Future Directions

The possibilities offered by NGS have clearly enhanced, or will enhance in the near future, our capacity to describe the pattern of diversity in three out of the four fields of research shown in Fig. 1, i.e., systematics of the Conoidea, diversity of prey, and

diversity of toxins. Even when small geographic distances are involved, apparent sympatry can actually correspond to bathymetrically separated species (Barghi et al. 2015c), and allopatric speciation thus cannot to be ruled out systematically. Nevertheless, the fact that ecological speciation occurs in conoideans is supported by a growing amount of data, in particular for closely\ related species with different diets that are found to co-occur in sympatry, or even in syntopy. Questions remain about the process leading to genetic differentiation in the presence of gene flow. How do barriers to gene flow, either pre- or postzygotic, appear, and how are they maintained? What is the exact role of toxin and prey diversification in the speciation process? How do these microevolutionary processes affect the general diversification pattern of Conoidea? Is there selection for species that are more prone to evolve new toxins (i.e., species selection for evolvability)? What is the role of prey specialization (or, on the contrary, expansion of dietary breadth) in the evolution of Conoidea? Other traits should not be neglected; for example, it has been shown that dispersal ability may influence diversification process in conoideans. Many hypotheses now bloom in the literature to explain conoideans and cone snails' evolution, but most remain to be thoroughly tested by describing the diversity patterns more precisely. Systematics still remains paramount to answer such questions, and as such effort should be put into describing species diversity and species relationships: only in the light of these data can the evolutionary hypotheses be tested.

Acknowledgments This review benefitted from the project CONOTAX, funded by the French ANR (ANR-13-JSV7-0013-01).

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Phylogeny of Annelida

18

Torsten H. Struck

Abstract

Annelida are typically characterized by the presence of segmentation and can be found in all habitats on the Earth. Traditionally regarded as being closely related to arthropods, with several very well-known toxic or venomous species, molecular data robustly placed them within Lophotrochozoa. Besides annelids, only one other taxon within Lophotrochozoa, Mollusca, is currently known to also contain toxic or venomous species. The phylogeny of Annelida has been controversially discussed since the recognition of Annelida as a taxon in the nineteenth century. However, recent phylogenomic studies have achieved tremendous progress in this respect. Based on these results, Annelida was split into two major clades, one clade (the Errantia) adapted to an errant mobile life and the other (the Sedentaria) which includes earthworms and leeches, to a more sessile, sedentary one. Finally, several morphologically aberrant annelid taxa are the first to branch off from the annelid stem lineage. Moreover, the nonsegmented Sipuncula (peanut worms) and Echiura (spoon worms) have to be placed within Annelida. Interestingly, the four taxa known to comprise toxic or venomous species are scattered throughout the tree. While Amphinomidae are part of the basal radiation, Glyceridae are placed within Errantia and the clitellate taxa *Eisenia* and Hirudinea are part of Sedentaria. Hence, the evolution of toxic or venomous species within Annelida most likely occurred independently.

Keywords

Annelida • Lophotrochozoa • Articulata • Errantia • Sedentaria • Glyceridae • Amphinomidae • Hirudinea • Eisenia

T.H. Struck (✉)
Department of Research and Collections, Natural History Museum, Oslo, Norway
e-mail: torsten.struck.zfmk@uni-bonn.de

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_15

Contents

Introduction

Annelida, the ringed or segmented worms, is an ancient and ecologically important animal taxon with over 22,000 described species occupying terrestrial, limnic, and marine habitats all around the world. Annelids can have body length of less than 1 mm or more than 3 m. As bioturbators, scavengers, and predators, they are important members of all ecosystems. Although some species can be found in the plankton throughout their entire life span, annelid species usually constitute a significant part of the endo- and epibenthos where they occupy almost every existing ecological niche in the marine environment (Purschke et al. 2014). Moreover, they are the most abundant macrofaunal organisms in the largest habitat on the planet, the deep sea. On the other hand, the vast majority of the limnetic and terrestrial species belong to only one group of annelids, the Clitellata (e.g., earthworms and leeches), which exhibit specific adaptations to the terrestrial life (e.g., Purschke 2002). Thus, annelids show a broad diversity of life strategies including tube-dwelling, burrowing, deposit-feeding, filter-feeding, predatory, blood-feeding, and parasitic strategies. Given this broad ecological and life history range, evolution of annelids has resulted in a high morphological diversity. Typically, annelids consist of segments containing coelomic cavities, nephridia, a pair of ganglia of the ventral nervous system, different types of muscles, and so-called parapodia bearing different kinds of chaetae. The presence of segmentally arranged chitinous chaetae is one of the key characters of annelids. Hence, Annelida comprises one of three major animal groups with segmentation. Besides the segments, annelids possess a prostomium and peristomium at the anterior end bearing different kinds of sensory organs, such as palps, antennae, and nuchal organs. In some annelid taxa, the pharynx is equipped with elaborate jaw elements for defense, prey catching, or blood feeding (e.g., Eunicida, Glyceridae (bloodworms), Nereididae, Gnathobdelliformes (jawed leeches)). At the posterior end, a pygidium is present, which might also bear sensory organs in the form of cirri. However, almost every character varies greatly among annelids, making ground pattern reconstruction a difficult task without a solid phylogenetic framework. For example, one of the key characters, segmentation, is virtually absent in some groups, e.g., Echiura (spoon worms), Sipuncula (peanut worms), or Diurodrilidae, a group of small interstitial annelids (Purschke et al. 2014).

Given this diversity of different life strategies, only very few annelid taxa and species are known to be toxic, either for defensive or predatory reasons. Amphinomidae (fireworms) bear calcareous chaetae instead of the typical chitinous ones. Upon contact, these chaetae break and can cause serious skin inflammation, for example, in humans (Borda et al. 2012). Upon attack, the earthworm *Eisenia fetida* releases a toxin with its coelomic fluid, which is hemolytic by inserting into cell membranes (Sukumwang and Umezawa 2013). On the other hand, parasitic leeches, Hirudinea, use different polypeptides preventing coagulation of the blood while feeding (Kvist et al. 2014). Bloodworms (Glyceridae) are thus far the only known annelids known to catch prey using venom (von Reumont et al. 2014b). As mentioned above, glycerids possess jaw elements. Specifically, they have four strong clawlike jaws, which are capable of injecting the venom due to a direct connection to the venom glands.

Phylogeny of Annelida

Phylogenetic Position of Annelida Within Bilateria

Traditionally, Annelida had been regarded as closely related to Arthropoda, which is also known as the Articulata hypothesis. The Articulata hypothesis was substantiated by characters such as a segmented body organization including a rope ladderlike nervous system and segment formation by a posterior growth zone, longitudinal muscles of the body wall in distinct bundles, and presence of mushroom bodies (Scholtz 2003). Interestingly, arthropods are among the bilaterian lineages from which several venomous animals are known, even by non-biologists. These include insects, centipedes, remipede crustaceans, scorpions, spiders, and ticks (Fry et al. 2009; von Reumont et al. 2014a). Given the number of toxic annelid and arthropod taxa, the Articulata hypothesis could support the view that there is a tendency to evolve venom in these animals.

In contrast, early molecular-phylogenetic studies based on 18S rRNA were not able to recover Articulata, but showed a closer relationship of Annelida to Mollusca and Brachiopoda (see Struck 2012). Studies including representatives of all lophophorate lineages (i.e., Brachiopoda, Phoronida, and Ectoprocta) confirmed these results with strong bootstrap support, and the name Lophotrochozoa was coined for this group of taxa. Lophotrochozoa is defined as including the last common ancestor of lophophorates, molluscs, and annelids and its descendants. The suitability of the 18S-rRNA data used in the first molecular studies regarding bilaterian relationships has been criticized. However, since then, an impressive array of different molecular markers had unequivocally supported a close relationship of Annelida to lophotrochozoan taxa. These markers comprised larger 18S rRNA datasets, 28S rRNA, Hox gene data, mitochondrial genomes, 19 nuclear protein-coding genes, microRNA data, and phylogenomic datasets consisting of more than 50 genes mostly derived from expressed sequence taqs (EST) libraries (see Struck

2012). Additionally, topology testing significantly rejected a close relationship of Annelida and Arthropoda. Thus, molecular data clearly support a placement of Annelida within Lophotrochozoa, though their position within Lophotrochozoa remains controversial (Edgecombe et al. 2011; Halanych 2004).

The term Spiralia is occasionally used as a synonym of Lophotrochozoa (Halanych 2004). However, the definition of Lophotrochozoa as provided above does not necessarily include the same set of taxa. The taxon Spiralia includes all animals with spiral cleavage (i.e., Annelida, Mollusca, Nemertea, Platyhelminthes, Gnathostomulida, and Entoprocta) (Edgecombe et al. 2011). Thus, depending on the position of the lophophorate taxa, Lophotrochozoa and Spiralia could be synonymous, Spiralia a subgroup of Lophotrochozoa, or vice versa. One challenge in reconstructing the lophotrochozoan/spiralian phylogeny is that many taxa (e.g., Platyhelminthes, Ectoprocta, Gastrotricha) are hampered by misleading molecular biases such as increased substitution rates in comparison to the other taxa in the analysis (also known as the long-branch attraction artifact) or compositional heterogeneity. Therefore, simply increasing the number of taxa is not enough to resolve this phylogeny, but thorough analyses of the data quality have to accompany the phylogenetic reconstruction. Recent phylogenomic studies utilizing more genes but also more sophisticated analytical strategies have achieved some progress in this respect (Fig. 1) (Nesnidal et al. 2013; Struck et al. 2014). These analyses showed that Lophotrochozoa is a subgroup of Spiralia and hence not synonymous with

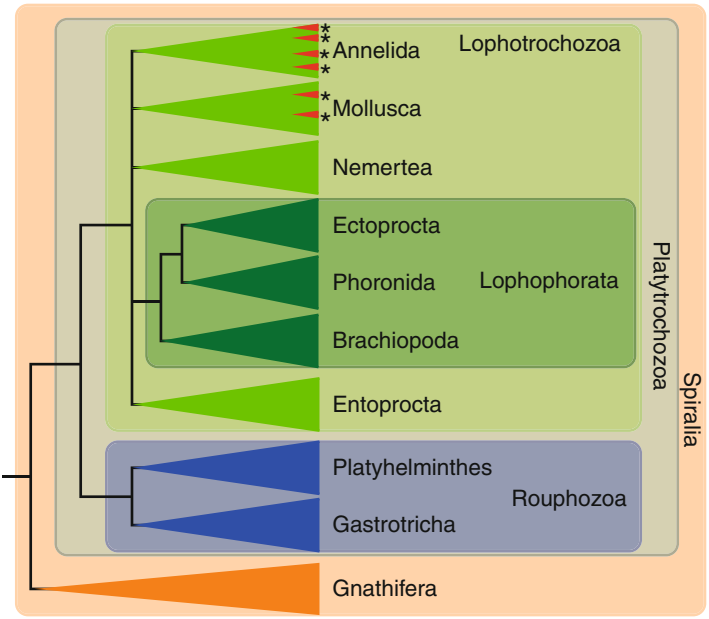

Fig. 1 Phylogeny of Spiralia based on recent phylogenomic studies (Nesnidal et al. 2013; Struck et al. 2014). *Red triangles* and *stars* indicate group of toxic or venomous species. Higher taxonomic units are highlighted

it. The first taxon to branch off in Spiralia is Gnathifera, which comprised among others the wheel animals "Rotifera." The Rouphozoa comprising Gastrotricha and Platyhelminthes are the sister of Lophotrochozoa, a clade named Platytrochozoa (Struck et al. 2014). The relationships within Lophotrochozoa are still inconsistent, but strong evidence is emerging that Lophophorata, comprising Ectoprocta, Brachiopoda, and Phoronida, is indeed monophyletic. The previously recognized clades Kryptrochozoa (i.e., Brachiopoda, Phoronida, Nemertea) and Polyzoa (i.e., Entoprocta, Cycliophora, Ectoprocta) could be attributed to misleading biases in the dataset due base composition heterogeneity (Nesnidal et al. 2013).

Summarizing these results, it is obvious that the molecular data unequivocally reject a close relationship of Annelida to Arthropoda. In contrast, Annelida are placed in a spiralian group also comprising Mollusca, Nemertea, and Lophophorata. Interestingly, besides annelids, molluscs are the only known taxon within this group with venomous species (i.e., cone snails and some cephalopods) (Fry et al. 2009). In contrast to the Articulata hypothesis, this position of Annelida does not support a view that there might be a tendency toward evolving venom in the lineage of Spiralia and independent convergent evolution of venomous animals seems more likely.

Monophyly and Taxon Composition of Annelida

Given the Articulata hypothesis, only very few characters like a foregut with dorsolateral folds and capillary chaetae of β-chitin in four groups supported the monophyly of Annelida. Some regarded even these to be present in the stem lineage of Articulata and placed arthropod taxa within Annelida (see Struck 2012). However, given the Lophotrochozoa hypothesis, several characters like segmentation with a praepygidial proliferation zone, a dorsal brain and a ventral nerve cord, longitudinal muscle bands, capillary chaetae of β-chitin in four groups, and nuchal organs support the monophyly of Annelida (see Purschke 2002). In contrast, recovery of the monophyly of Annelida within Lophotrochozoa was difficult in earlier molecular-phylogenetic studies (see McHugh 2000, 2005). However, recent studies with increased numbers of taxa and genes and more sophisticated methods consistently recovered the monophyly of Annelida even with strong nodal support (see Struck 2012). Additionally, it could be shown that Annelida exhibit a unique mitochondrial gene order (Golombek et al. 2013). However, monophyly of Annelida based on molecular data required the adjustment of the taxon composition of Annelida. Traditionally, Annelida comprised Clitellata and Polychaeta (Fauchald 1977); now, other taxa, formerly considered as separate phyla, have to be included within the annelid radiation (i.e., Myzostomida, Siboglinidae, Echiura, Sipuncula) (Halanych et al. 2002).

For myzostomids, flat-bodied marine ectocommensals or parasites of echinoderms, a close relationship to Annelida or placement within polychaetes had generally been assumed, even though the body plan of Myzostomida is unique due their ectocommensalic/parasitic life history (see Struck 2012). Several features support an inclusion of Myzostomida within Annelida: parapodia with chitinous chaetae

(aciculae and hooks) and cirri, chaetogenesis, similarities in larval development via trochophore- and nectochaeta-like larvae, ultrastructure of the nervous system, hypertrophied axial pharynx, and serial arrangement of protonephridia (Lanterbecq et al. 2008). However, analyses of molecular data (i.e., EF1α, 18S, and 28S rRNA) seemed to support the latter by placing Myzostomida closer to taxa like Platyhelminthes or Ectoprocta. However, again careful analyses of the molecular data showed that the results were due to biased data (Bleidorn et al. 2009). Molecular data of Myzostomida which is not, or is less, affected by increased substitution rates such as myosin II heavy chain and mitochondrial proteins, as well as mitochondrial gene order data, strongly support a close relationship of Myzostomida and Annelida. Hox gene data also substantiate this position. Recently, phylogenomic studies based on hundreds of genes were also able to place Myzostomida within Annelida (Hartmann et al. 2012; Weigert et al. 2014).

Siboglinidae have had a controversial taxonomic history. Upon description of the first species, they were compared to hemichordates. However, they were also regarded as aberrant annelids. Deuterostome affiliations were substantiated by some by a dorsal nerve cord, radial cleavage, and tripartite body with prosoma, mesosoma, and a modified metasoma, whereas others placed them within protostomes, specifically close to Annelida, due to the possession of a ventral nerve cord, spiral cleavage, and metameric segmentation and homology of their chaetae with uncini of polychaetes (see Struck 2012). The discovery that the specimens investigated till then were incomplete due to the lack of the segmented ophistosoma (Webb 1969) revealed that Siboglinidae possess a segmented body organization. Furthermore, ultrastructural analyses further supported the homology of the chaetae of Siboglinidae with ones of Annelida (Bartolomaeus 1995). Finally, morphological cladistic analyses also supported a closer relationship of Annelida and Siboglinidae (Rouse and Fauchald 1997), as did different molecular data: elongation factor 1α, cytochrome oxidase c subunit I, hemoglobin, 18S and 28S rRNA, multi-gene analyses, mitochondrial genomes, and phylogenomic data (see Struck 2012; Weigert et al. 2014). Additionally, topology testing significantly rejected an exclusion of Siboglinidae from Annelida (Struck et al. 2007) and analyses of combined morphological and molecular data also placed Siboglinidae within Annelida (Zrzavy et al. 2009).

Echiura are unsegmented marine worms and were grouped together with Annelida throughout the nineteenth century. Several features support a close affinity of Echiura with Annelida: a typical annelid-like trochophore larva, chaetogenesis and ultrastructure of the chaetae, structure and organization of the blood vascular system, and development and ultrastructure of spermatozoa (see Struck 2012). However, de Quatrefages (1847) transferred them to Gephyrea, which also comprised Sipuncula, Sternapsidae, and Priapulida, a group supposed to bridge the gap between annelids and echinoderms. As Echiura also lack any sign of segmentation as adults as well as nuchal organs, Echiura were excluded from Annelida, though a close relationship to Annelida or Articulata was still advocated (Clark 1969). The exclusion of Echiura was further substantiated by morphological cladistic analyses of Annelida (Rouse and Fauchald 1997). However, the distinction between primary or secondary absence of character traits such as segmentation in cladistic analyses

based on morphological data might be problematic. Moreover, the development of the nervous system in Echiura reveals traces of an ancestral segmentation pattern (Hessling 2003). First support from molecular data for an inclusion of Echiura within Annelida was derived from elongation factor 1α data (McHugh 1997). As for siboglinids, to date ample molecular data support the inclusion of Echiura within Annelida (see Struck 2012). Topology tests significantly rejected an exclusion of Echiura from Annelida (Struck et al. 2007).

Finally, Sipuncula are relatively large, unsegmented worms (more than 90 % are >5 mm) burrowing in marine sediments. Sipuncula were considered to be closely related to Mollusca based on the so-called molluscan cross during spiral cleavage (Scheltema 1993). On the other hand, on the basis of developmental features, Rice (1985) suggested a close affinity to Annelida. Additionally, the early nervous system development showed signs of a posterior growth zone similar to annelids with some metameric patterns (Kristof et al. 2008). In contrast to Siboglinidae and Echiura, first molecular-phylogenetic studies were inconclusive with respect to the position of Sipuncula as closer relationships to either Mollusca, Annelida, or Mollusca plus Annelida were suggested (see Struck 2012). However, increasing the amount of molecular data for both Sipuncula and other lophotrochozoan taxa has achieved solid placement of Sipuncula within Annelida (see Struck 2012; Weigert et al. 2014). Moreover, topology tests significantly rejected an exclusion of Sipuncula from Annelida (Dordel et al. 2010).

Internal Relationships of Annelida

Throughout the nineteenth century, three groups were generally recognized within Annelida: Polychaeta, Oligochaeta, and Hirudinea. Polychaeta comprised all marine annelids, which were henceforth referred to also as marine bristle worms. Oligochaeta comprised both earthworms and microdrilids and Hirudinea, the leeches, acanthobdellids, and branchiobdellids. In the nineteenth century, Polychaeta had been traditionally divided into two major groups, which however were known under different names and with different taxon compositions. Generally, one group contained polychaetes such as Amphinomidae, Eunicida, or Phyllodocida but also other polychaetes which were predominantly characterized by a more or less carnivorous lifestyle. The other clade contained all remaining polychaetes like Spionidae, Chaetopteridae, Opheliidae, or Sabellidae, which were characterized by a microphagous diet (see Struck 2012). Finally, de Quatrefages (1866) established the most influential systematic scheme, dividing polychaetes by their life style. Polychaeta was split into Annelidae erraticae (later named Errantia) and Annelidae sedentariae (later named Sedentaria). The names clearly reflected their mode of living as either being errant, more vagile, worms or sedentary, more sessile, ones, but the scheme itself was based on the presence or absence of distinct body regions also known as tagmatization (de Quatrefages 1866). Errantia comprised polychaetes like Amphinomidae, Eunicida, and Phyllodocida lacking any obvious body regions. On the other hand, Sedentaria were characterized by the presence of distinct body

regions. With some modifications, the classification scheme of de Quatrefages (1866) was adopted in the following century. In summary, Errantia comprised Eunicida, Phyllodocida and Amphinomidae, and Sedentaria the remaining polychaetes (Struck 2012). For a detailed account of the early history of the phylogeny of polychaetes and Annelida, please refer to Struck (2012).

Michaelsen (1928–1932) grouped Oligochaeta and Hirudinea together as Clitellata and it has been accepted for a long time now that Hirudinea is placed within Oligochaeta (Erséus 2005). Since Michaelsen (1928–1932), most annelid researchers have generally treated Clitellata and Polychaeta as two separate taxa within Annelida (e.g., Dales 1963; Fauchald 1977). Support for the monophyly of Clitellata is overwhelming based on both morphological and molecular data, but the monophyly of Polychaeta was not similarly supported (Purschke 2002). For example, the morphological cladistic analyses of Rouse and Fauchald (1997) strongly supported the monophyly of Clitellata, but support for the monophyly of Polychaeta was weak, as none of the supporting characters was free of homoplasy.

In the middle of the last century, the classification scheme of polychaetes based on de Quatrefages (1866) came under heavy criticism for showing only arbitrary groupings reflecting mode of living rather than evolutionary history and being valuable only for practical purposes (Dales 1963, 1977). Consequently this classification system of Errantia and Sedenataria was completely abandoned by the end of the last century, but a new scheme had not yet arisen. Instead, polychaete families were grouped together into several orders of equal rank as no morphological characters unequivocally supported any higher groupings (Fauchald 1977; Struck 2012). Therefore, the phylogeny of Polychaeta was regarded as unresolved.

At the end of the last century, Rouse and Fauchald (1997) tried a new approach to reconcile the different polychaete families into higher taxonomic groupings based on morphological data. Using a cladistic method considering all data simultaneously instead of only a single or a few characters, they employed two coding strategies by coding either 124 morphological characters as absent/present or 55 characters as multistate, respectively, for 80 polychaete families. Moreover, different strategies were used to analyze the data, for example, by weighing the characters differently or by excluding symbiotic, pelagic, interstitial, and poorly known taxa, as these taxa often show highly aberrant morphologies or the states are not known yet in the case of poorly described species leading to instable placements of these taxa in the reconstructed trees. Based on these analyses, Rouse and Fauchald (1997) proposed as sister group relation of Clitellata and Polychaeta. Polychaeta was further split into Scolecida comprising Arenicolidae, Capitellidae, Opheliidae, Orbiniidae, Paraonidae, and Cossuridae as well as Palpata consisting of the remaining polychaetes. The scolecidan taxa are earthworm-like and hence the name Scolecida from the Greek scolex for worm. Palpata comprised all palp-bearing polychaetes and the possession of palps was the supporting character for this clade. Palpata was divided into Canalipalpata and Aciculata. Aciculata consisted of Amphinomidae, Eunicida, and Phyllodocida and, hence, was in its composition similar to Errantia. Of all clades proposed by Rouse and Fauchald (1997), Aciculata was the best supported with respect to morphological characters, besides the presence of the name-giving

aciculae, an internalized supporting chaetae, other characters substantiated this clade (Rouse and Fauchald 1997). Moreover, it contained both polychaete groups with toxic or venomous species. Most of the remaining polychaetes grouped together as Canalipalpata characterized by the presence of peristomial grooved palps, but some of the taxa listed above with aberrant morphologies were placed as *incertae sedis* (Rouse and Fauchald 1997). While this scheme of annelid phylogeny became generally accepted in a short time, it was also instantly strongly criticized for the exclusion Clitellata from polychaetes, inconsistencies in character reconstructions, as well as their results from the different analytical strategies and lack of structural integrity for reconstructed stem species (Bartolomaeus et al. 2005; Struck 2012; Westheide 1997).

In parallel to the analyses of Rouse and Fauchald (1997), the inclusion of Clitellata within polychaetes was explicitly proposed in the last decade of the twentieth century based on both molecular data and functional morphology. Nielsen (1995) suggested incorporating Clitellata within polychaetes, based on similarities to Capitellidae. Westheide (1997) discussed the direction of evolution within polychaetes based on the functional morphology of the coelom and coelothels and concluded that Clitellata is a highly derived polychaete subtaxon with many of the differences to polychaetes, such as the reduction of appendages, being related their unique reproduction. However, the polychaete subtaxon forming the sister taxon to Clitellata could not be determined based on morphological data. In summary, based on morphological data, it remains uncertain whether or not to place Clitellata within polychaetes. Neither monophyly of Polychaeta nor a sister group relationship between Clitellata and a polychaete subtaxon is convincingly supported.

The first molecular-phylogenetic studies, based on elongation factor 1α, placed Clitellata within polychaetes. All subsequent molecular-phylogenetic studies also congruently recovered the placement of Clitellata within polychaetes utilizing 18S rRNA, 28S rRNA, histone H3, U2 snRNA, cytochrome oxidase subunit I, 16S rRNA mitochondrial genomes, as well as a steadily increasing number of taxa (see Struck 2012). However, as for the morphological data, a polychaete sister group of Clitellata could not be determined, as nodal support was weak, and the placement of Clitellata differed between the analyses. Moreover, these molecular-phylogenetic studies also seemed to still suffer from low-resolution power as judged by nodal support (see McHugh 2005). Nonetheless, increasing the number of both taxa and position had, in general, a positive effect on the reconstruction on the annelid phylogeny (see Struck 2012). Due to these additional genes, monophyly of Annelida, including the taxa discussed above, was consistently recovered, and topology testing significantly rejected monophyly of Scolecida, Palpata, Canalipalpata and Aciculata (Struck et al. 2007). Moreover, the congruence between the results of different studies increased, but nonetheless nodal support remained low (Struck et al. 2007, 2008; Zrzavy et al. 2009), precluding any robust conclusions of the phylogeny with respect to polychaete families and the position of Clitellata (Struck 2012).

Simulation studies by Struck et al. (2008) showed that annelid phylogeny could be resolved using more data. Moreover, Dordel et al. (2010) revealed that amino acid

data would be better suited to achieve this goal than nucleotide data. Therefore, instead of using specific genes, a phylogenomic study of Annelida was conducted using a dataset of nearly 48,000 amino acid positions and data coverage of 41.7 % per taxon (Struck et al. 2011). This large and reasonably well-covered dataset resulted in a well-supported phylogeny with several basal nodes showing bootstrap values of 70 or higher, even up to the maximal value of 100. Interestingly, the results of this phylogenomic approach were congruent in many parts to the studies of Struck et al. (2008) and Zrzavy et al. (2009), which were based on many fewer genes. One of the major differences was the position of the Amphinomidae, which was part of Errantia in Struck et al. (2011), instead of being part of the basal radiation. A study concentrating on the effect of paralogous genes, which have been grouped together erroneously as orthologous genes, revealed that even in the presence of 231 genes, a single such misplacement has the potential to mislead the placement of the affected taxon, given certain circumstances such as low taxon coverage for the corresponding taxon (Struck 2013). For example, due to one instance of such erroneously grouped paralogous genes, Amphinomidae was placed within Errantia (Struck 2013). A follow-up phylogenomic study further increasing both taxon sampling as well as number of genes (>600 genes) found results similar to the phylogenomic analyses of Struck et al. (2011) except that Amphinomidae was part of the basal annelid radiation and Orbiniidae of Sedentaria (Fig. 2, Weigert et al. 2014). Moreover, in this study all basal relationships within the annelid phylogeny were supported by significant bootstrap values of 99 or 100 and misleading effects on the reconstruction by paralogy, contamination, long-branch heterogeneity, or other biases could be excluded. Hence, the century-old debate about the phylogeny of Annelida including the placement of Clitellata within polychaetes could finally be settled using massive amounts of molecular data (Fig. 2, Struck et al. 2011; Weigert et al. 2014).

Oweniidae, Mageloniidae, Chaetopteridae, Amphinomidae, and Sipuncula have been found to be part of the basal annelid radiation (Fig. 2). Hence, of the two polychaete taxa with toxic or venomous species, Amphinomidae and Glyceridae, one was placed as part of this basal radiation closely related to Sipuncula, which previously have been regarded as its own phylum. The remaining annelids split into two well-supported groups. One clade comprised Eunicida and Phyllodocida and the other one the remaining annelids including Clitellata and Echiura (Fig. 2). As the taxon compositions of these two clades were very similar to the taxon compositions of the traditional Errantia and Sedentaria concepts, respectively, Struck et al. (2011) resurrected these names for these two clades with some modifications. Errantia was defined as the clade comprising all descendants of the last common ancestor of Eunicida, Phyllodocida, and all organisms or species that share a more recent common ancestor with these two taxa than with Clitellata and Spionidae, whereas Sedentaria was oppositely defined as the clade comprising Clitellata, Echiura, Spionidae, and all organisms or species that share a more recent common ancestor with these three taxa than with Phyllodocida or Eunicida (Struck 2012). Moreover, Struck (2011) named the clade comprising Errantia and Sedentaria Pleistoannelida as it comprises most of

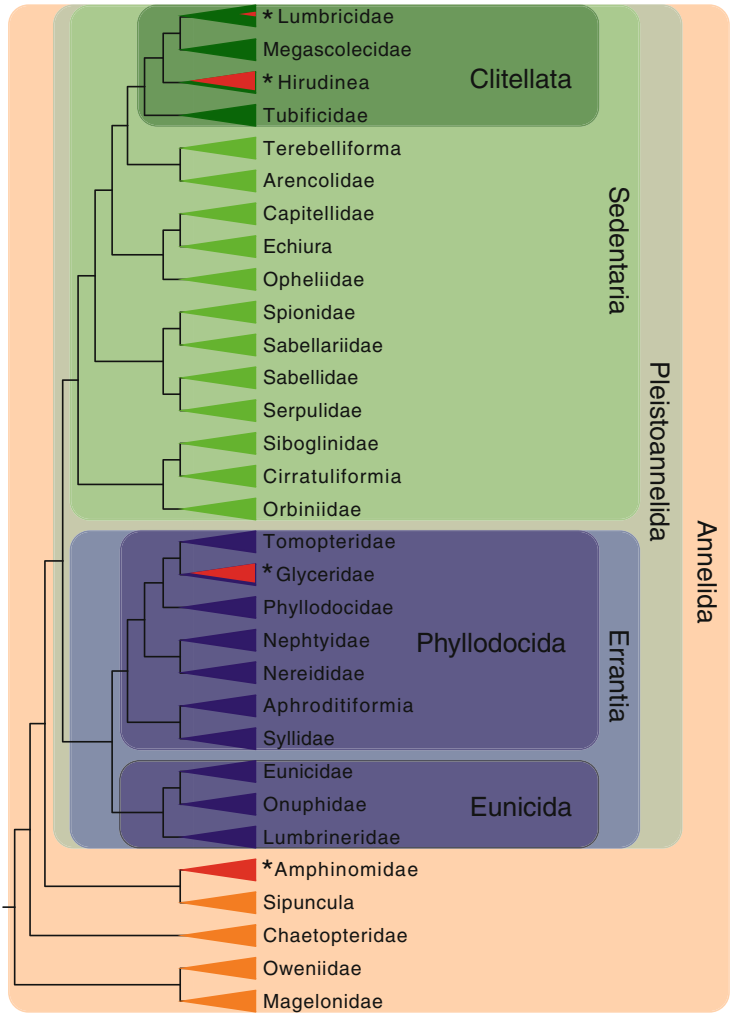

Fig. 2 Phylogeny of Annelida based on the phylogenomic study of Weigert et al. (2014). As Fig. 1 *red triangles* and *stars* indicate group of toxic or venomous species. Higher taxonomic units are highlighted

the recent annelid biodiversity and defined it by the last common ancestor of Sedentaria and Errantia and all the descendants of that ancestor. The second polychaete taxon with toxic or venomous species, Glyceridae, was placed within the errant annelid taxon Phyllodocida (Fig. 2, Weigert et al. 2014). However, although relationships within Phyllodocida were strongly supported in the analyses of Weigert et al. (2014), taxon representation of Phyllodocida was still too limited to allow definite conclusions about the sister group of Glyceridae within

Phyllodocida. Based on morphological data, the most likely sister group of Glyceridae is Goniadidae (Rouse and Fauchald 1997).

Within Sedentaria, Clitellata were placed within a clade consisting also of Arenicolidae, Terebelliformia, Capitellidae, Echiura, and Opheliidae, as sister to Arenicolidae/Terebelliformia (Fig. 2), with very strong support (Weigert et al. 2014). Moreover, this clade is congruent with the placement of Clitellata in previous phylogenomic studies with a much more limited taxon sampling of Annelida (e.g., Dordel et al. 2010). Additionally, in the analyses of Struck et al. (2008), Clitellata were part of a congruent clade. This study was based only on 18S and 28S rRNA data, but with a much more comprehensive taxon sampling than the phylogenomic studies (Dordel et al. 2010; Struck et al. 2011; Weigert et al. 2014). The placement of Clitellata within polychaetes also means that Polychaeta is synonymous with Annelida and should be therefore abandoned as a taxon name.

Two of the four groups with toxic or venomous species, Hirudinea and the genus *Eisenia* (Lumbricidae), are clitellates (Fig. 2). Within Clitellata, Hirudinea is part of a clade that also comprises the classical earthworms (Lumbricidae) as well as all other megadrilid taxa and Enchytraeidae (Erséus 2005). Hence, both clitellate groups with toxic or venomous species are part of this group. However, as in Lumbricidae only species of the genus *Eisenia* are so far known to release toxins, convergent evolution of the toxic or venomous character trait is more likely than a common origin with several losses. This is further substantiated by the fact that there are differences in the toxins. *Eisenia* uses the protein lysenin, which is hemolytic and might also play a role in the innate immune reaction, while hirudineans release a complex mixture of anticoagulant polypeptides (von Reumont et al. 2014b).

Conclusions and Future Directions

Of the four annelid taxa with toxic or venomous species, two are placed within Clitellata as part of the sedentary annelids, but not as sister groups to each other (Fig. 2). Glyceridae is part of Errantia and Amphinomidae part of the basal radiation of Annelida. Hence, convergent evolution of venomous and toxic species even within Annelida seems very likely.

With respect to the phylogeny of Annelida, and especially considering the evolution of venomous annelids, future research should be directed to three more specific tasks. First, what are the lophotrochozoan sister group of annelids and also the other lophotrochozoan taxon Mollusca with venomous species? Second, while the phylogenetic position of Hirudinea, *Eisenia* and Amphinomida has been settled in the last few decades, the position of Glyceridae within Phyllodocidae remains to be determined. Third, within Amphinomidae and Glyceridae, the ingroup relationships of the genera and species to each other are not yet resolved. This is an especially important task for Glyceridae, as glycerid species are emerging as model systems for studying venoms in annelids (see von Reumont et al. 2014b).

Cross-References

- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins
- ▶ Systematics of Cephalopods
- ▶ Toxicity in Cephalopods

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Systematics of Cephalopods

19

A. L. Allcock

Abstract

Cephalopoda is an extremely diverse class of mollusks that has been evolving since the Cambrian. The extant lineages arose in the late Silurian and diverged into Nautiloidea and Coleoidea in the mid-Palaeozoic. Nautiloidea is represented by a handful of Recent species only. In contrast, Coleoidea has diverged into two superorders, Decapodiformes and Octopodiformes, which together comprise around 800 Recent species. The relationships among orders of Decapodiformes are not well understood, and molecular systematics has failed to provide much resolution, although there is some evidence for a sister-taxon relationship between Spirulida, the ram's horn squid, and Sepiida, the cuttlefishes. A sister-taxon relationship between Bathyteuthida and Oegopsida is well established. The relationships among Octopodiformes are better understood. The vampire squid is placed in a separate order, and all other octopods are placed in Octopoda. Within Octopoda there are well-understood clades: Octopoda is divided into Cirrata and Incirrata; Incirrata is further divided into Argonautoidea and Octopodoidea. Several lineages of cephalopods have been evolving independently for a long time: for example, Spirulida, represented by a single extant species, appears to have diverged from other groups 150 million years ago, nautiloids appear little changed since 300 million years ago, and Vampyromorphida, also represented by a single extant species, appears little changed since 200 million years ago. In contrast, several groups, for example, the sepiids, appear to have undergone recent radiations.

Keywords

Evolution • Paleontology • Molecular phylogenetics • Coleoidea • Nautiloidea • Octopodiformes • Decapodiformes • Squid • Cuttlefish • Octopus

A.L. Allcock (✉)
School of Natural Sciences, National University of Ireland Galway, Galway, Ireland
e-mail: louise.allcock@nuigalway.ie

P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3_8

Contents

Introduction

The molluscan class Cephalopoda arose in the Cambrian (Young et al. 1998; Kröger et al. 2011). Arguably the most diverse of all molluscan lineages, cephalopods have adapted to occupy multiple niches in the marine environment, from estuarine bays to shallow neritic shelf seas, to the open ocean, and to benthic abyssal plains.

Cephalopods emerge in the fossil record in Cambrian strata, although there is some dispute over which fossils represent stem cephalopods, a not uncommon problem in paleontology where fossils are rare and where preservation of soft parts is usually far from complete. Reviewing available literature, Kröger et al. (2011) suggested that stem cephalopods were present in the early Cambrian (e.g., *Tannuella*, a mollusk with a chambered shell) and that the earliest undisputed cephalopod fossil is *Plectronoceras cambria*, whose chambered shell probably facilitated buoyancy control as seen in modern nautiloids.

Kröger et al. (2011) place the origin of the lineage that contains all modern (as well as some extinct) cephalopods in the late Silurian. They suggest that this lineage arose from the orthocerids, cephalopods with straight but chambered external shells, and that, in the mid-Palaeozoic, it diverged into the two recognized subclasses that have Recent representatives, Nautiloidea and Coleoidea. The nautiloids found in the Indo West Pacific today are markedly similar to their Palaeozoic ancestors and are widely recognized as living fossils. They retain the external chambered shell and have simple "pinhole camera" eyes (without a lens). In contrast, the coleoids have diverged substantially. Molecular divergence estimates (Kröger et al. 2011) place the initial divergence of coleoids into two extant (Decapodiformes and Octopodiformes) and three extinct (Phragmoteuthida, Belemnitida, and Diplobelida) lineages in the Permian, although undisputed fossils of the extant coleoid lineages are only found in much more recent strata. Further divergence of Decapodiformes and Octopodiformes then appears to have occurred throughout the Jurassic and Cretaceous to provide the variety of forms present in the ocean today (Fig. 1).

Decapodiformes comprises about 500 Recent species in between five and seven orders (see Table 1), depending on taxonomic opinion, whereas Octopodiformes comprises approximately 300 species in two orders, with all but one species within the order Octopoda. Within the general bauplan of mantle, head, and arms, the diversity of forms is remarkable: animals may be streamlined or robust,

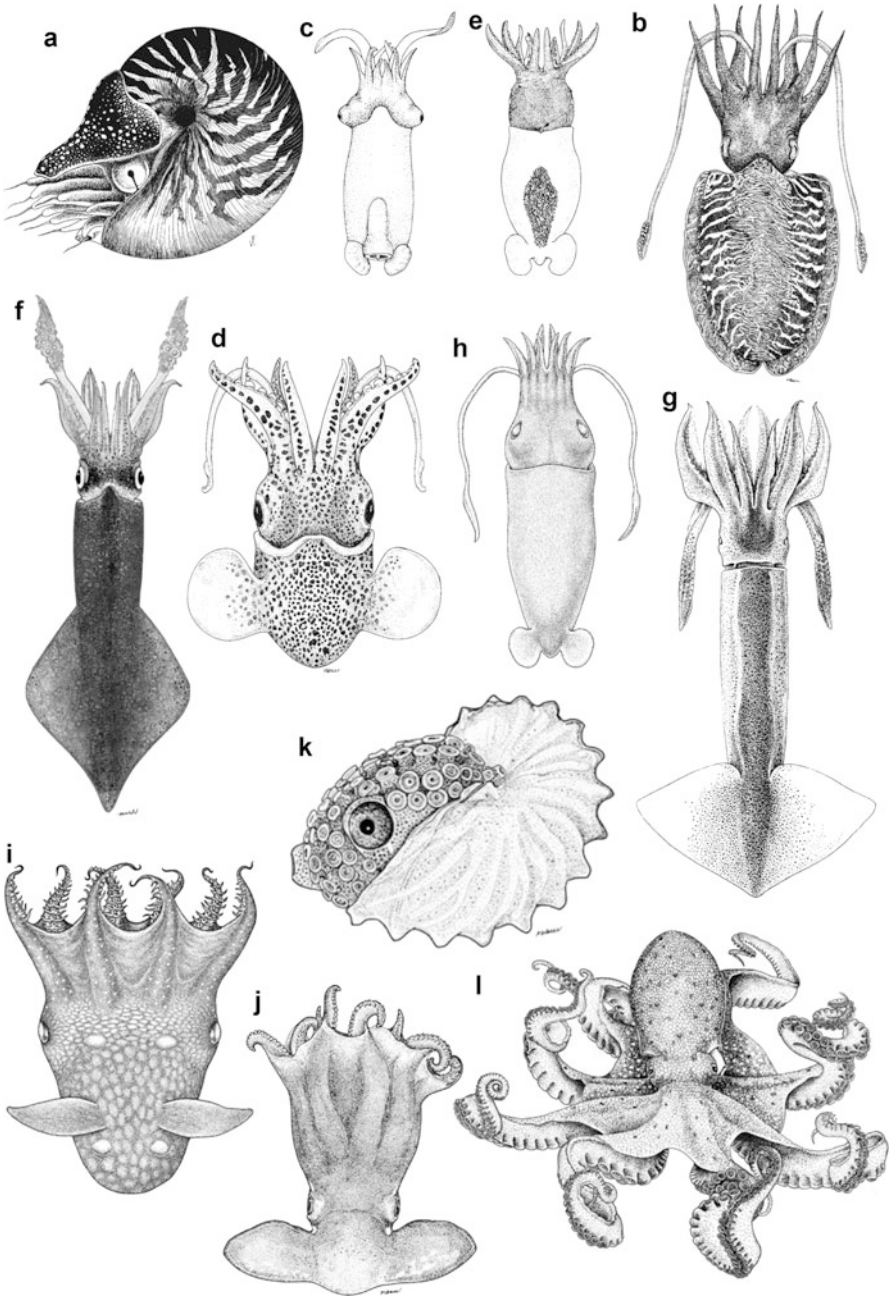

Fig. 1 Representatives of major extant lineages of cephalopods. (**a**) Nautiloidea, *Nautilus macromphalus*; (**b**) Sepiida, *Sepia officinalis*; (**c**) Spirulida, *Spirula spirula*; (**d**) Sepiolida, *Sepiola affinis*; (**e**) Idiosepiida; (**f**) Myopsida, *Loligo vulgaris*; (**g**) Oegopsida, *Ommastrephes bartramii*;

Table 1 Major lineages within Decapodiformes. A minimal number of defining characters are provided. For additional characters, see Young et al. (2012a)

Order	Common name	Number of species	Defining characters	Nomenclatural notes
Spirulida	Ram's horn squid	1	Shell as coiled phragmocone, without cornea	Some authors treat at family level as Spirulidae
Sepiida	Cuttlefishes	~120	Shell as flattened phragmocone, with cornea	Some authors treat at family level as Sepiidae
Sepiolida	Bob-tailed squids and bottle-tailed squids	~70	Shell as rudimentary gladius, with cornea	
Myopsida	Squids, often "neritic squids" because of their habitat preferences	~50	Shell as gladius, with cornea	
Idiosepiida	Pygmy squids	~6	Shell as gladius, with cornea, with adhesive organ	Some authors treat at family level as Idiosepiidae
Oegopsida	Squids, often "oceanic squids" because of their habitat preferences	~230	Shell as gladius, without cornea, with carpal locking apparatus	
Bathyteuthida	Squids	~6	Shell as gladius, without cornea, without carpal locking apparatus	Some authors treat at superfamily level as Bathyteuthoidea

dorsoventrally flattened or not, and may be adapted to benthic, demersal, or pelagic lifestyles. All coleoids, in contrast to nautiloids, have an internalized shell, but this takes many forms. Cuttlefishes (order Sepiida, superorder Decapodiformes) have a dorsoventrally flattened phragmocone, the "cuttlebone." Ram's horn squids (order Spirulida, superorder Decapodiformes) have a small internal calcareous-chambered open planispiral shell which is used in buoyancy. Most other Decapodiformes have a pen-like gladius, but this may be reduced or absent in some groups. Among Octopodiformes, the vampire squid (which is actually more closely related to octopuses and is the sole representative of the order Vampyromorphida) has a thin gladius, cirrate octopuses have reasonably robust internalized cartilaginous shells,

Fig. 1 (continued) (**h**) Bathyteuthida, *Bathyteuthis abyssicola*; (**i**) Vampyromorphida, *Vampyromorpha infernalis*; (**j**) Cirrata, Cirroctopodidae; (**k**) Argonautoidea, *Argonauta hians*; (**l**) Octopodoidea, Octopodidae (Figures reproduced with kind permission of the FAO, Rome, from Jereb and Roper (2005, 2010), and Jereb et al. (2014))

and incirrate octopuses tend to have small vestigial internal shell remnants, the "stylets." There is some relationship between shell type and habitat/lifestyle: for example, the buoyancy controls provided by the phragmocones of cuttlefishes and ram's horn squids allow them to exploit the water column, the cartilaginous shells of cirrate octopuses support the fins they use for swimming, and the gladius reflects the streamlined form of squids that facilitates their speed in their pelagic environment. In many cases, the diversity among groups is so great that establishing which groups are most closely related has been a difficult task for systematists and evolutionary biologists.

Nautiloidea

Just a handful of extant nautiloid species exist today, although there is some dispute over the actual number. They are considered to be living fossils, shell morphology having changed little since the late Carboniferous (Wani and Mapes 2010), and they exhibit many of the cephalopod characteristics thought to be plesiomorphic, including an external chambered shell and pinhole eyes as detailed above. Biogeographically, modern nautiloids are restricted to the Indo West Pacific, but nautiloids were more widespread in their earlier history, being both abundant and distributed worldwide from the Jurassic to the Miocene (Teichert and Matsumoto 1987).

Today, they are found on reefs at approximately 100–700 m depth, where they are both scavengers and predators. They are slow growing, and are estimated to reach maturity after about 15 years, and have a life span in excess of 20 years (Dunstan et al. 2011). In captivity, at least, they are slow to reproduce, laying very few eggs throughout the year (Arnold et al. 1990), and this *k*-selected life history strategy makes them particularly susceptible to overfishing (in support of the ornamental shell trade) to which they are subjected through much of their range.

Modern nautiloids are placed in two genera, *Nautilus* and *Allonautilus*, discriminated by differences in gill structure and the male reproductive system, among other characters (Ward and Saunders 1997), within a single family, Nautilidae. The currently recognized species are *Allonautilus scrobiculatus* (Lightfoot, 1786), known from Papua New Guinea and surrounding islands; *Nautilus pompilius* Linnaeus, 1758, originally described from Ambon in Indonesia, with an extensive distribution and possibly comprising a species complex; *Nautilus belauensis* Saunders, 1981, from Palau; *Nautilus macromphalus* Sowerby, 1849, from New Caledonia; *Nautilus repertus* Iredale, 1944, from Western Australia; and *Nautilus stenomphalus* Sowerby, 1849, from the Great Barrier Reef. *Allonautilus perforatus*, which is known from drift shells in Bali, Indonesia, is probably synonymous with *A. scrobiculatus.*

Molecular genetic work on *N. pompilius* across a wide geographic range has shown several populations to be extremely divergent (Sinclair et al. 2007; Bonacum et al. 2011; Williams et al. 2012). Samples from Vanuatu, Fiji, and American Samoa were more closely related to *N. macromphalus* than to other *N. pompilius*, and species from Eastern Australia and Papua New Guinea were clearly divergent

from those from the Philippines, Indonesia, Western Australia, and Palau. Bonacum et al. (2011) suggested that several of these populations actually represented phylogenetic species but did not tackle the nomenclature. Sinclair et al. (2007) found further divergence between *N. pompilius* samples from the Great Barrier Reef and the Coral Sea separated by small geographic distances, and Williams et al. (2012) also found this pattern, as well as separation of these populations from samples from Western Australia, the Philippines, and Indonesia.

Other species not currently considered valid have been described, and Young (2010) provides a complete list of nominal species. Many of these names do not, in fact, refer to nautilids and some are *nomen nudum,* but a few are nomenclaturally valid and may be applied in the future, particularly to widely separated populations that are found to merit specific status. Thus, it is important to note that nautilid systematics is in a state of flux. Given their status as living fossils and the fact that they have been evolving independently for hundreds of million years, nautilids may prove very interesting in comparative studies of venom with other cephalopods and indeed other mollusks. Therefore, knowledge of their more recent radiations and the current diversity within this group may be important for venom studies.

Coleoidea: Decapodiformes

There are seven main groups of decapodiforms (Table 1). Herein they are all recognized as separate orders, but differing opinions exist as to their ranks. In two of these groups, Spirulida and Sepiida, the shell is a phragmocone (i.e., chambered as in nautiloids, although internal as in all coleoids). However, the form of the phragmocone is highly divergent between Spirulida and Sepiida. Spirulida comprises the single species *Spirula spirula* Linnaeus, 1758. It is a midwater species that seems to be most abundant over bottom depths of 1,000–2,000 m (e.g., continental slopes or slopes associated with volcanic islands). *Spirula* has a long fossil record, extending back to the latest Jurassic (Kröger et al. 2011). Often also referred to as a "living fossil," its lineage is estimated to have diverged from that of other decapodiforms 150 mya (Warnke et al. 2011). Species of the order Sepiida are characterized by the presence of a cuttlebone. Sepiida comprises more than 100 species of cuttlefishes that live in continental shelf waters although they may extend onto the slope to depths of about 600 m. They are present along tropical and temperate coasts of the world including Australasia, Asia, Africa, and Europe but are totally absent from North and South America. Sepiids have a benthic or demersal lifestyle, are short lived, and spawn large eggs for an extended period once they reach maturity. Cuttlefishes are dorsoventrally flattened as is their internal phragmocone, the cuttlebone, which runs the length (or most of the length) of the body. The phragmocone of spirulids is superficially very different: the planispiral calcareous phragmocone is situated rostrally and occupies a much smaller portion of the body.

The temptation to unite these two groups on morphological grounds is resisted not only because of superficial differences but also because the phragmocone is a

plesiomorphic structure. Nonetheless, the wall structure of their phragmocones is similar and differs from that of extinct coleoids such as Belemnitida and non-coleoid ectocochleate cephalopods (Doguzhaeva 1996; Young et al. 1998). Furthermore, Spirulida and Sepiida share other morphological characters such as statolith shape and the structure of the tentacular clubs (Clarke and Maddock 1988; Young et al. 1998).

Young and Vecchione (1996) conducted a cladistics analysis of extant coleoid cephalopods based on 50 morphological characters. Their analysis neither resolved relationships within Decapodiformes nor found a sister-taxon relationship between Sepiida and Spirulida. In fact, in their analysis, all decapodiform taxa branched as a polytomy from a single node in a strict consensus tree. Molecular studies have often also failed to recover deep cephalopod relationships. However, two studies (Strugnell et al. 2005; Lindgren and Daly 2007) have found support for a sister-taxon relationship between Spirulida and Sepiida. These studies used different nuclear genes (Pax6, octopine dehydrogenase, rhodopsin versus 18S rRNA), but it should be noted that bootstrap support values for the 18S rRNA tree were relatively low (66 %). Nonetheless, conflicting topologies have not been found either. The major problem with molecular studies to date is that they have failed to yield topologies with notable bootstrap or posterior probability support on deep nodes.

Khromov (1998) discussed the biogeography of Recent sepiids (which are absent from the Americas) in light of paleoceanography and concluded they had a very recent origin in the Old World, a conclusion that fits with the available fossil data (Young et al. 1998). Naef (1921–1923) concurred, suggesting similarly recent origins for idiosepiids and sepiolids. Although a close relationship between these taxa is far from certain, if correct, these conclusions have implications for the length of time the various branches have been diverging when considering comparative studies of their venom.

Naef (1921–1923) studied fossil, morphological, and embryological evidence and recognized a close relationship between Spirulida and Sepiida but also considered these taxa to be closely united with Idiosepiida and Sepiolida. Idiosepiida is a monogeneric taxon of pygmy squids. These tiny squids are circa 2 cm long as adults, and the synapomorphic character for the group is an adhesive organ on the dorsal surface of their mantle, which they use to attach to sea-grass blades or other algae in their inshore habitat. Sepiolida is a more diverse order, comprising the bob-tailed and bottle-tailed squids. These small round cephalopods have broad posteriorly placed fins and may be benthic, demersal, or pelagic. Naef treated Spirulida, Sepiida, Idiosepiida, and Sepiolida as families and placed them together in the superfamily Sepioidea. Although sepiolids have a gladius rather than a phragmocone, Naef (1921–1923), who conducted meticulous embryological studies, concluded that a phragmocone anlage could be deduced from the form of the embryological shell sac. Naef did not conduct embryological studies on *Idiosepius* but concluded that a similar form would be seen. Naef (1921–1923) noted other similarities between Sepiolida and Idiosepiida, including the similarity of the shell, and the presence of an adductor pallii medialis and suggested that Sepiolida developed from an "*Idiosepius*-like predecessor." Strugnell et al. (2005) did find some support from nuclear genes

for a sister-taxon relationship between *Idiosepius* and sepiolid species, particularly when just the third codon positions were used in the analysis. Note that the nomenclature is somewhat confusing, since more recent authors (e.g., Young et al. 2012a) use the name Sepioidea to describe a clade containing just Sepiolida and Sepiida, although this two-taxon grouping is not widely accepted.

The neritic squids, Myopsida, and the oceanic squids, Oegopsida, the latter sometimes assumed to include the bathyteuthids, have often been combined into the taxon Teuthida. However, support for this taxon is equivocal. Superficially, the morphology of Myopsida and Oegopsida is similar. The shell (or gladius) is similar, they share the same long streamlined body, and they have similar tentacular clubs. However, myopsids share several characters with sepiids and sepiolids that oegopsids do not have, the most notable of which is the presence of a cornea. Thus, despite their superficial similarity to oegopsids, some authors consider myopsids to be more closely related to sepiids and sepiolids. Once again, molecular data have failed to resolve this issue. In many cases, phylogenies have even failed to recover these orders as monophyletic. This is not believed to reflect confused systematics as these orders are well defined morphologically. Furthermore, these orders tend to resolve as well-supported clades in studies based on multiple nuclear genes (e.g., Strugnell et al. 2005; Lindgren and Daly 2007; Strugnell and Nishiguchi 2007; Lindgren 2010; Lindgren et al. 2012). Molecular studies do recognize the close relationship between Bathyteuthida (which many authors do not treat at order level) and Oegopsida, but this has anyway long been recognized. Naef (1921–1923) suggested that Bathyteuthidae possesses characters primitive for all Oegopsida and *Bathyteuthis* has historically been placed in Oegopsida.

Multigene phylogenies (Strugnell et al. 2005; Strugnell and Nishiguchi 2007; Lindgren 2010; Lindgren et al. 2012), combined morphological and molecular analyses (Lindgren et al. 2004), and analyses based on mitochondrial genome rearrangement (Allcock et al. 2011) have failed to resolve deep relationships among Decapodiformes lineages (for review see Allcock et al. 2015). It is likely these will only be resolved by genomic studies (Albertin et al. 2012). Fortunately, relationships within some of these orders are better understood.

Sepiida

Cuttlefishes are perhaps best known for their remarkable camouflage, signaling patterns, and behavior, all of which are relatively easy to study, given their presence in shallow coastal waters. There are just three genera in a single family Sepiidae within the order Sepiida, with species distributed unevenly among the genera. There are two species within the genus *Metasepia*, which is characterized by a reduced cuttlebone. Species of *Metasepia* are only found around Australia and in the Western Pacific. There are seven species with the genus *Sepiella*, which is characterized by a subcutaneous gland opening through a pore between the fins, the function of which is unknown. *Sepiella* is also found in the Pacific with species as far north as Japan and Korea and as far south as northern Australia, but the distribution of this genus also extends westward with two species known from the Mozambique coast and a

third species extending down the west coast of Africa from Mauritania to Namibia (Barratt and Allcock 2012).

All other species of sepiid are grouped in the large (with more than 100 species) genus *Sepia*. Khromov (1998) attempted to diagnose subgroups within the genus *Sepia*. He defined and provided keys to six species complexes within the genus: *Hemisepius*, *Acanthosepion*, *Sepia* sensu stricto, *Anomalosepia*, *Rhombosepion*, and *Doratosepion*. He further allocated all (at that time) recognized species to one of these species complexes and provided keys to the species within each complex. There have been no large-scale molecular studies with extensive taxon sampling of sepiids to date to verify these subdivisions. Nonetheless, Yoshida et al. (2010), in a relatively small-scale study, did find molecular support for *Doratosepion* and *Acanthosepion*. However, molecular phylogenies have also highlighted an unexpected relationship between *Sepia officinalis* and *Sepiella* (Bonnaud et al. 2006; Yoshida et al. 2010; Lindgren et al. 2012), suggesting that our current understanding of generic level relationships is not totally correct.

The genus *Sepia* has the widest distribution of all the sepiid genera, perhaps not surprisingly, since it contains very many more species than the other genera. It occurs off the coasts of Europe, Asia, Africa, and Australasia; however, as mentioned above, it is absent from the coasts of North and South America. Khromov (1998) analyzed biogeographic patterns in sepiids. He found greatest diversity in the Indo West Pacific, which was home to 91 species, 86 of which were endemic to the region. In contrast, the Northeast Atlantic, including the Mediterranean Sea, was home to just nine species, five of which were endemic. He concluded that the northern and southern limits of the family were governed by temperature. Low diversity in parts of Indonesia was attributed to poor faunal knowledge of that region; however, Khromov (1998) found clear evidence of a decrease in species numbers from inshore waters of the Pacific and Indian Oceans to more remote island habitats, with sepiids absent from Hawaii, the Seychelles, and the Chagos Archipelago. The inability of sepiids to colonize habitats separated by deep water likely reflects their lack of dispersal phase: adults lay large eggs that give rise to miniature benthic hatchlings which inhabit the same habitat as the adult phase. Molecular investigations of island endemics from places such as Guam and the Marshall islands may therefore show evidence of founder populations.

The earliest fossil sepiids are found in US deposits but their identification is disputed. *Voltzia* from the Upper Jurassic and *Actintosepia* from the Cretaceous have been considered not to be sepiids by some authors (e.g., Waage 1965). However, of the five genera reported from the Eocene (*Archaeosepia*, *Belosepia*, *Pseudosepia*, *Sepia*, *and Stenosepia*), two are known from US deposits (Roeleveld 1972; Khromov 1998), but only *Sepia* is known from more recent strata (Roeleveld 1972). Hence, it is likely that low water temperatures, particularly in the western Atlantic in the Oligocene, led to the extinction of sepiids from the Americas. Although the genus *Sepia* later radiated out of Europe, Roeleveld (1972) suggests that temperatures would have been too low on the only suitable routes for recolonization (i.e., via the Bering Straits or from Europe to Greenland via the Faroe Islands and Iceland). Khromov (1998) proposed that *Sepia* subsequently

radiated from the Mediterranean Ocean, spreading to the developing Indian Ocean in the Oligocene and then radiating throughout Southeast Asia. He suggests that the African fauna and that of the Japan Sea (i.e., those species on the periphery of the range) are therefore the youngest in evolutionary terms. There is considerable concordance between the subgeneric classification and geographic location, presumably reflecting these different radiations.

Sepiolida

There are two families within Sepiolida: the eponymous Sepiolidae and Sepiadariidae. Sepiadariidae is a small family of bottle-tailed squids in just two genera: *Sepioloidea* and *Sepiadarium*. Neither genus has a shell remnant, and the members of the genera are colorful small squids that live on the seafloor in mostly shallow tropical seas of the Indo West Pacific. *Sepioloidea* is known from Australia, Indonesia, and New Zealand, with the distribution of one species extending along submarine ridges as far as Easter Island (Reid 2005, 2009). *Sepiadarium* has a slightly broader distribution with species occurring from South Australia northward through the Pacific to Japan and westward to east India and Sri Lanka.

In contrast, the family Sepiolidae is more diverse, comprising 16 genera divided into three subfamilies. Sepiolids are small-rounded squids, with a rudimentary gladius, which may be absent, and posterior fins, not dissimilar in overall shape to the sepiadariids. Members of two of the subfamilies, Rossiinae and Sepiolinae, are benthic, while members of Heteroteuthinae are pelagic or benthopelagic. All heteroteuthins have a large visceral photophore, and Naef (1921–1923) believed this subfamily to be the most derived form. Unfortunately, because of the very delicate shell in this group, there is no fossil evidence with which to consider evolutionary pathways.

The relationships among genera are not completely clear. Young (2007) placed *Sepiola*, *Euprymna*, *Inioteuthis*, *Rondeletiola*, and *Sepietta* in Sepiolinae; *Heteroteuthis*, *Nectoteuthis*, *Stoloteuthis*, *Iridoteuthis*, *Amphorateuthis*, and *Sepiolina* in Heteroteuthinae; and *Rossia*, *Austrorossia*, *Neorossia*, and *Semirossia* in Rossiinae. This reflects Naef's (1921–1923) placements with the exception that *Sepiolina* is placed in Heteroteuthinae rather than Sepiolinae. Young (2007) left *Choneteuthis* unplaced, noting its similarity to *Sepiolina* but also the differences of *Sepiolina* to other Heteroteuthinae. Young (2007) also highlighted the presence of an as-yet undescribed subfamily whose affinities are not clear, specimens of which are currently only known from fish stomachs.

A multigene molecular phylogeny of Decapodiformes (Lindgren et al. 2012) suggests that our understanding of sepiolid relationships may not yet be complete. The phylogeny supported monophyly of Sepiadariidae and confirmed Sepiadariidae and Sepiolidae to be sister taxa. This study, which included representatives of eight genera of Sepiolidae (Rossiinae, *Rossia*; Heteroteuthinae, *Heteroteuthis*, *Stoloteuthis*, *Sepiolina*; Sepiolinae, *Euprymna*, *Sepiola*, *Sepietta*, *Rondeletiola*), found *Stoloteuthis* as sister taxon to *Rossia* and consequently did not support the monophyly of Heteroteuthinae. Only two species of Rossiinae were included, but they did group as sister taxa. The 13 included species of Sepiolinae formed a clade,

but within this clade, the genera *Sepiola* and *Sepietta* were not monophyletic, although included members of the genus *Euprymna* did form a clade. Groenenberg et al. (2009) also failed to recover monophyletic genera within Sepiolinae in a study using the cytochrome oxidase subunit I (COI) barcode gene. Importantly, Groenenberg et al. (2009) highlighted the presence of several misidentified sequences on GenBank that could confound future studies: see Groenenberg et al. (2009) and Lindgren et al. (2012) for details.

Idiosepiida

Represented by the single genus *Idiosepius* in the family Idiosepiidae, pygmy squids also have an Indo West Pacific distribution. However, the delimitation of species is not clear, and researchers conducting comparative studies on venom should be aware of this. The currently known specimens probably comprise a single species (*Idiosepius minimus*) off the coasts of Africa, a species endemic to Australia (*Idiosepius notoides*), and at least four species in the Indo West Pacific whose precise ranges and overlaps have not been elucidated (*I. picteti*, *I. thailandicus*, *I. paradoxus*, and *I. pygmaeus*). *Idiosepius biserialis* is probably a junior synonym of *I. minimus* and confined to African coasts, and species treated under this name from the Indo West Pacific likely refer to *I. thailandicus*. DNA barcoding has helped clarify the distribution of some species (Byern et al. 2012), but much further work is required.

Myopsida

Myopsid squids are found in neritic zones where they inhabit pelagic or demersal waters. They number, in total, about 50 species and there have been several major rearrangements of myopsid systematics. The currently accepted classification has all species except one in the family Loliginidae, with a second monospecific family Australiteuthidae restricted to northern Australia and Papua New Guinea. Eleven genera, and a number of subgenera, are currently recognized as valid within Loliginidae. Intriguingly, most of these genera appear to affiliate with particular geographic regions, much as the subgenera of sepiids do. *Loligo*, *Afrololigo*, and *Alloteuthis* are associated with Europe and Africa. *Doryteuthis*, *Lolliguncula* and *Pickfordiateuthis* are associated with the Americas. *Heterololigo* is associated with the northern Pacific. *Loliolus*, *Uroteuthis*, and *Photololigo* are associated with the Indo West Pacific. The exception is *Sepioteuthis* which is distributed in the western Atlantic, the Mediterranean, the Indo West Pacific, and around the coasts of Australia as far south as Tasmania. This distribution led Brakoniecki (1986) to suggest that *Sepioteuthis* is a Tethyan relic, and molecular data (Anderson 2000; Sales et al. 2013) support this, indicating *Sepioteuthis* to be the sister taxon to all other loliginids. Anderson (2000) further suggested subsequent dispersal from the Indo West Pacific to East Pacific American waters, possibly along the continental shelf on the northern periphery of the Pacific, and then further radiation in the Americas, possibly driven by the uplift of the isthmus of Panama. This could explain the sister-taxon relationship between *Heterololigo* and *Doryteuthis* found in molecular studies and the sister-taxon relationship between *Doryteuthis opalescens* (Berry, 1911) in American Pacific waters and *Doryteuthis pealeii*

(Lesueur, 1821) in American Atlantic waters. To date, no molecular studies have included Australiteuthidae.

There is marked genetic structure among populations of some loliginid species (Dai et al. 2012; Sales et al. 2013; Zheng et al. 2012), possibly indicative of a number of cryptic species or ongoing speciation in these taxa.

Oegopsida

Oegopsid squids are found in the open ocean, and this taxon comprises the largest order of cephalopods with more than 200 species in 24 families and 69 genera. Seven families are monospecific (Architeuthidae, *Architeuthis dux* Steenstrup, 1857; Batoteuthida, *Batoteuthis skolops* Young and Roper, 1968; Joubiniteuthidae, *Joubiniteuthis portieri* (Joubin, 1912); Ancistrocheiridae, *Ancistrocheirus lesueurii* (d'Orbigny, 1842); Psychroteuthidae, *Psychroteuthis glacialis* Thiele, 1920; Lepidoteuthidae, *Lepidoteuthis grimaldii* Joubin, 1895; Thysanoteuthidae, *Thysanoteuthis rhombus* Troschel, 1857), and a further three families are monogeneric (Promachoteuthidae, Magnapinnidae, Pholidoteuthidae). The families with greatest diversity are Cranchiidae (13 genera, circa 60 species), Ommastrephidae (11 genera, circa 22 species), Onychoteuthidae (7 genera, circa 25 species), and Gonatidae (4 genera, circa 19 species). Relationships among families are not well understood, but some families are believed to be closely associated.

Young and Vecchione (2004) propose four groupings of multiple families. They highlight loss of the true tentacular club as a feature shared by Batoteuthidae, Chiroteuthidae, Joubiniteuthidae, Magnapinnidae, Mastigoteuthidae, and Promachoteuthidae. However, they note that monophyly of this group is far from certain. Lindgren et al. (2012), in a total evidence molecular study, found five of these families formed a clade that also included Pholidoteuthidae; Promachoteuthidae was not included in the study. This clade was not strongly supported, but a subclade containing Batoteuthidae and Chiroteuthidae received bootstrap support >90 %, and a subclade containing Joubiniteuthidae, Mastigoteuthidae, and Magnapinnidae received bootstrap support >50 %.

Small mesopelagic squids in the families Ancistrocheiridae, Enoploteuthidae, Lycoteuthidae, and Pyroteuthidae may be related (Young and Vecchione 2004). Lindgren et al. (2012) found that members of Enoploteuthidae and Pyroteuthidae formed a clade, which had good bootstrap support >90 %. However, members of Lycoteuthidae fell together, but no support was found for a relationship between Lycoteuthidae and any other family. Ancistrocheiridae was not included in the study.

Histioteuthidae and Psychroteuthidae share the same tentacular club structure, and their relationship is supported by 100 % bootstrap support in a total evidence molecular study (Lindgren et al. 2012).

The fourth family-level relationship is among Lepidoteuthidae, Octopoteuthidae, and Pholidoteuthidae. There is no single uniting character for this grouping (Young and Vecchione 2004), and it is not supported as a monophyletic group by molecular work. Nevertheless, there is some bootstrap support (>50 %) for a relationship between Lepidoteuthidae and Octopoteuthidae (Lindgren et al. 2012).

Relationships within some of the four most diverse families are better understood than others. Cranchiidae is divided into two subfamilies: Cranchiinae and Taoniinae. While there have been no dedicated molecular studies on Cranchiidae, Lindgren et al. (2012) included three cranchiins and seven taoniins and found the family and both subfamilies to be monophyletic.

In contrast, a molecular study of Gonatidae, using three mitochondrial genes and including 14 species representing all four genera, found all genera, except *Eogonatus* which is monospecific, to be polyphyletic (Lindgren et al. 2005).

Similar issues have been found within Onychoteuthidae. Early molecular work (Bonnaud et al. 1998) suggested that the genera *Onychoteuthis* and *Moroteuthis* were in need of taxonomic revision. Subsequently Wakabayshi et al. (2007) used DNA barcoding to show that adults identified as belonging to the genus *Moroteuthis* fell in a clade with paralarvae known as *Onykia* (a genus where adults were unknown). However, to complicate matters further, *Onykia* does not appear to be monophyletic, since *Onykia carriboea* Lesueur, 1821, has fallen outside the main *Onykia* clade in molecular studies (Lindgren 2010; Lindgren et al. 2012) and resolved as sister taxon to *Ancistroteuthis lichtensteini* (Férussac [in Férussac and d'Orbigny], 1835).

Ommastrephidae has been divided into three subfamilies, Illicinae, Ommastrephinae, and Todarodinae which can be diagnosed on a few simple characters such as the sucker seriation on the dactylus of the tentacular club and whether or not photophores are present (Young et al. 2012b). A study using two mitochondrial genes and including 15 species in ten genera provided support for these divisions, recovering all the subfamilies as monophyletic in nearly all analyses (Wakabayashi et al. 2012). However, within Todarodinae, two genera (*Todarodes* and *Nototodarus*) were found to be polyphyletic, casting doubt on the characters used to separate them.

Bathyteuthida

This small group of deep-water squids currently comprises six species: three in the genus *Chtenopteryx* (family Chtenopterygidae) and three in the genus *Bathyteuthis* (family Bathyteuthidae). A molecular study including two *Chtenopteryx* species and three *Bathyteuthis* species recovered both genera as monophyletic and also as sister taxa (Lindgren et al. 2012).

Coleoidea: Octopodiformes

Octopodiformes comprises approximately 300 species in two orders, with all but one species within Octopoda. The order Vampyromorphida is represented by the single species *Vampyroteuthis infernalis* Chun, 1903 – the enigmatic vampire squid, widely recognized as a living fossil. It inhabits mesopelagic depths in the temperate to tropical zones of the world's oceans. Until recently, there was much debate as to whether the vampire squid was a representative of Decapodiformes or

Octopodiformes. However, its placement in Octopodiformes is confirmed by morphological evidence from hatchlings (Young and Vecchione 1999) and from embryological evidence of octopods (Boletzky 1978–1979, 2006). Molecular evidence has given conflicting results, but an increasing body of molecular evidence also supports the placement of Vampyromorphida in Octopodiformes (see, e.g., Yokobori et al. 2007; Strugnell and Nishiguchi 2007; Allcock et al. 2011). Kröger et al. (2011) place the earliest vampyromorph fossils in the late Triassic/early Jurassic, suggesting that this lineage has been evolving independently for about 200 million years.

The order Octopoda comprises two suborders, Cirrata (or Cirrina) and Incirrata (or Incirrina), and molecular evidence provides strong support for monophyly of these groups and for a sister-taxon relationship between them (Carlini et al. 2000; Strugnell et al. 2004, 2005, 2014; Lindgren et al. 2012). The earliest evidence of these taxa in the fossil record is found from Late Cretaceous deposits (Tanabe et al. 2008; Fuchs et al. 2009), although divergence time estimates (Kröger et al. 2011) place their separation in the Late Jurassic.

Cirrata comprises the finned octopods, which tend to have a gelatinous body and a deep web. The name Cirrata derives from the cirri that extend down the arms alongside the suckers. The fins are supported by an internal cartilaginous shell and the animals use these to swim, and while some finned octopods are primarily benthic, they may also be demersal or entirely pelagic.

Cirrata was reviewed by Collins and Villanueva (2006). In their systematic section, they followed the proposals made by Piertney et al. (2003), based on molecular work involving a single mitochondrial gene, that Cirrata comprises four families. Revising some of the existing taxonomy and clarifying the status of some difficult taxa, Collins and Villanuevea (2006) suggested the following families were valid: Cirroteuthidae for the pelagic genera *Stauroteuthis*, *Cirroteuthis*, and *Cirrothauma*; Grimpoteuthidae for the genera *Grimpoteuthis*, *Cryptoteuthis*, and *Luteuthis*; Opisthoteuthidae for the genus *Opisthoteuthis*; and Cirroctopodidae for the genus *Cirroctopus*. However, other arrangements are also followed. For example, Vecchione et al. (2014) place the genera in three families as follows: Opisthoteuthidae (*Cirroctopus*, *Grimpoteuthis*, *Luteuthis*, *Opisthoteuthis*, *Cryptoteuthis*), Cirroteuthidae (*Cirroteuthis*, *Cirrothauma*), and Stauroteuthidae (*Stauroteuthis*). They also note the morphological similarity between Cirroteuthidae and Stauroteuthidae (united as Cirroteuthidae by Collins and Villanueva), which have similar body shape, long cirri, and a secondary web, but which differ markedly in shell shape. Although these classifications are not widely different, further molecular work with additional markers would be extremely useful.

Incirrata comprises the benthic octopuses familiar from shallow waters, as well as some more unusual groups. Young et al. (1998) discussed the unusual "oral-end-down" habit of benthic octopuses, which mostly crawl on the seafloor using their arms. They noted that the brain of *Vampyroteuthis* indicates that it is capable of processing complex chemotactile signals from the arms and wondered whether the arms played some important role in *Vampyroteuthis* or its ancestors that might have facilitated oral exploratory behavior of the seafloor so as to lead to the evolution of benthic octopods. In fact, recent work (Hoving and Robison 2012) shows that

vampire squids are detritivores and use their retractile filaments to accumulate food in a sticky matrix which is then passed to the mouth, so oral exploration of the benthos in a hypothetical ancestor is not an unreasonable proposition. However, this mode of feeding also raises interesting questions as to the role of venom in vampyromorphs, and comparisons between vampyromorphs and octopods would be interesting from an evolutionary point of view.

Incirrata is divided into two superfamilies: Argonautoidea, comprising the genera *Haliphron*, *Argonauta*, *Ocythoe*, and *Tremoctopus*, each placed in their own family, and Octopodoidea, comprising all other genera of incirrate octopods. The argonautoid families are unusual and highly diverse but are united by an unusual feature: that males have a detachable hectocotylus. The only known species of *Haliphron* inhabits deep waters around the world. It shows exceptional sexual dimorphism, with females reaching a total length of 2 m versus about 30 cm in males. The four *Tremoctopus* species, known as blanket octopuses, have extensive but thin webs. They float in the upper layers of subtropical and tropical oceans. They are also sexually dimorphic, with males about 5 % the size of females, which can reach more than a meter in total length. The four *Argonauta* species are also found in the upper layers of subtropical and tropical oceans. They are unique in that the female secretes a calcareous shell, in which it lives and lays its eggs. It is from the delicate nature and shape of the shell that the animal gets its common name "paper nautilus." Males are also dwarf and have been reported associated with salps. The single known species of *Ocythoe* is also found in upper water layers but of temperate oceans. The males are also dwarf.

Several molecular studies confirm Argonautoidea as sister group to all other incirrate octopuses (Strugnell et al. 2004, 2014; Lindgren et al. 2012). Naef (1921/1923) suggested that Alloposidae and Tremoctopodidae were closely related to one another, as were Argonautidae and Ocythoidae, based on the structure of the hectocotylized arm. Bizikov (2004) supported this arrangement, but based on the structure, or absence, of the stylets (shell remnants). Subsequent molecular work (Strugnell and Allcock 2010), based on several mitochondrial genes, supported this arrangement.

Argonautoidea has been hypothesized to have a benthic ancestry, because of the morphological resemblance of species to benthic octopuses in Octopodoidea (Naef 1921–1923). Relevant characters include the absence of fins and cirri, and a well-developed frontal lobe system, and evidence of corneas (Young et al. 1998).

Octopodoidea is easily the most speciose group of Octopodiformes, and relationships within it are still not well understood, although some advances have been made in recent years. Until recently, all benthic Octopodoidea were placed in the family Octopodidae, with the four valid pelagic genera distributed in the families Amphitretidae (*Amphitretus*), Vitreledonellidae (*Vitreledonella*), and Bolitaenidae (*Bolitaena*, *Japatella*) and often combined into a suborder Ctenoglossa on the basis of the structure of their radulae. However, early molecular work (Carlini and Graves 1999; Carlini et al. 2001) had suggested that the family Octopodidae was not monophyletic, and further evidence for this was provided in a study showing that the pelagic genera were a derived branch within Octopodidae, leading the authors to

Table 2 Major lineages within Octopodiformes

Order	Suborder	Superfamily	Included families
Vampyromorphida			Vampyroteuthidae
Octopoda	Cirrata		Cirroctopodidae, Cirroteuthidae, Grimpoteuthidae, Opisthoteuthidae
	Incirrata	Argonautoidea	Alloposidae, Argonautidae, Ocythoidae, Tremoctopodidae
		Octopodoidea	Amphitretidae, Bathypolypodidae, Eledonidae, Enteroctopodidae, Megaleledonidae, Octopodidae

suggest these genera had neotonous origins (Strugnell et al. 2004). This topology has since been recovered in multiple studies (Strugnell et al. 2008, 2014; Lindgren et al. 2012), which led Strugnell et al. (2014) to propose a revised taxonomy based on a molecular study utilizing three nuclear and four mitochondrial genes and including representatives of 25 Octopodoidea genera as well as *Vampyroteuthis* and representatives of the argonauts and cirrates. They combined the four pelagic genera into a single family Amphitretidae, with the genera placed within the subfamilies Amphitretinae, Vitreledonellinae, and Bolitaeninae. They placed the genus *Bathypolypus* in a family of its own, Bathypolypodidae. *Eledone* and *Aphrodoctopus*, two genera with uniserial suckers and heteromorphic arm tips in males, were combined in the family Eledonidae. Southern Ocean and deep-sea octopuses with uniserial suckers were combined in the family Megaleledonidae. The origins of this clade were explored by Strugnell et al. (2008), who showed that changing environmental conditions in the mid-Miocene led to strengthening of the thermohaline circulation and the consequent spreading of Antarctic bottom water northward, allowing radiation of a clade of octopuses out of Antarctica. *Muusoctopus*, *Enteroctopus*, *Sasakiopus*, and *Vulcanoctopus* were placed in a new family Enteroctopodidae. All other genera remained in the family Octopodidae.

A summary of octopod higher-level systematics is provided in Table 2. Although our understanding of octopus systematics has improved substantially in recent years, there are still many species whose generic affinities remain unclear. The tightening of the diagnosis of the genus *Octopus*, which for many years had contained a large number of widely divergent species, left many species without a generic placement. The work of Strugnell et al. (2014) shows that currently available molecular markers are well suited to solving such problems.

Conclusions

Cephalopoda is a particularly interesting class from an evolutionary point of view and therefore provides extensive opportunities for comparative studies. It is highly divergent, with widely differing body forms and species that inhabit widely differing environments. It is not particularly speciose, making comparative studies across the whole group a real possibility. Furthermore, it contains species on long branches,

which have been evolving independently for hundreds of millions of years such as the nautiluses, *Spirula* and *Vampyromorpha*, as well as groups that have undergone recent radiations such as the sepiids, possibly *Idiosepius*, and some myopsid genera. Our understanding of deep phylogenetic relationships is still poor and may only be solved by genomics, but suitable molecular markers do exist to solve many of the remaining taxonomic issues at more shallow nodes, and progress in these areas is likely to be rapid in the coming years, dependent only on sampling opportunities and resources.

Cross-References

► Toxicity in Cephalopods

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P. Gopalakrishnakone, A. Malhotra (eds.), *Evolution of Venomous Animals and Their Toxins*, Toxinology, DOI 10.1007/978-94-007-6458-3