Parasitology Research Monographs 10

Giovanni Benelli · Heinz Mehlhorn
Editors

Mosquito-borne Diseases

Implications for Public Health

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Parasitology Research Monographs 10

Series editor

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Chapter 1
Introduction I: Personal Insights in the Problem: What Remains to Be Done

Giovanni Benelli

Despite decades of extensive research efforts, mosquitoes (Diptera: Culicidae) still play a crucial role among vectors of medical and veterinary importance (Benelli 2015). Indeed, besides the widely known malaria burden, which led to 6.8 million deaths averted globally since 2001 (Benelli and Beier 2017), dengue virus poses at risk 3900 million people in 128 countries (Bhatt et al. 2013). In addition, lymphatic filariasis is still ranked among the most important neglected tropical diseases, and—at the same time—Zika virus outbreaks in the Americas and the Pacific are attracting high public health attention (Petersen et al. 2015; Benelli and Romano 2017), due to the arboviral connection with fetal microcephaly and neurological complications, with special reference to the Guillain–Barré syndrome (Oehler et al. 2014; Benelli and Mehlhorn 2016).

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To effectively manage mosquito populations, a rather wide number of control routes have been attempted, including classic applications of chemically synthesized pesticides, wide employ of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), as well as the development of eco-friendly formulations of novel insecticides (covering also nanostructured materials) (Benelli 2016, 2018) and mosquito repellents and field testing of biological control agents and biotechnological tools (Benelli et al. 2016; Bourtzis et al. 2016). However, only few of these tools have been approved by the World Health Organization Vector Control Advisory Group (WHO - VCAG), and there is an urgent need to validate several of them through epidemiological evidences (Benelli and Beier 2017).

The present books present authoritative book chapters written by experts in the field of mosquito vectors and mosquito-borne diseases, to provide an updated overview of the current mosquito research scenario. Key questions formulated—and sometimes addressed—in the present book focus on mosquito morphology, biology, genetics, ecology, and control.

Some of the most relevant ones about mosquito biology and ecology are: which is the updated vector status of mosquitoes widespread in Europe? Which mosquito species are endangering public health in India and other Asian countries? Are mosquitoes able to transmit HIV? What do we really know about the potential carcinogenic action of some pathogens and parasites vectored by several mosquito species?

Concerning mosquito control, crucial issues to deal with are: which are the main drawbacks arising from the use of chemical pesticides? How outbreaks of mosquito-borne diseases can be prevented by proper vector control operations? Do herbal and microbial products represent a challenging solution to develop novel mosquito repellents and insecticides of commercial interest? Which strategies are currently adopted during army field activities to protect humans from mosquito bites? Do long-lasting insecticide-treated textiles have a promising potential in the fight against mosquitoes?

Overall, all these questions urgently need a competent reply from public health experts, epidemiologists, parasitologists, biologists, and entomologists. As co-Editor of the present book, I am aware that this *Parasitology Research Monograph* cannot fully reflect the high diversity of the ideas and new insights rapidly growing in the field of mosquito vector research. Furthermore, I hope that this book will significantly contribute to boost research and applications on successful mosquito control strategies, along with an improved knowledge about the impact of vector biology and ecology, on the success of real-world mosquito control programs.

Conflict of Interest The author declares no competing interests.

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Chapter 2
Introduction II: Why Are Mosquitoes and Other Bloodsuckers Dangerous? Adaptations of Life Cycles and Behavior

Heinz Mehlhorn

The present book deals with recent aspects of bloodsucking arthropods such as mosquitoes, biting flies, fleas, midges, ticks, etc., which exist much longer on earth than the present generations of humans, who are targeted by these nasty contemporaries. Bloodsuckers are not only troublesome but also dangerous due to their ability to transmit agents of diseases such as prions, viruses, bacteria, fungi, and/or parasites, which might even lead to death (Mehlhorn 2016a, b). The transmission may occur during sucking lymph fluid or by oral uptake of blood, when injecting their mouthparts into the blood vessels (e.g., mosquitoes) or sucking at little blood "lakes" being produced by peculiar cutting mouthparts (e.g., tabanids, ticks) (Figs. 2.1 and 2.2).

All known bloodsuckers run their life cycles successfully not only in their typical endemic regions, where they are supported by the slightly but constantly increasing phenomena of global warming, but constantly enlarge their territory due to an enormously increasing globalization process including the daily transportation of goods, persons, and animals from one end of the world to the other. The present book will deal with some selected examples, which give an impression, how vulnerable the world population is. However, it is comforting that epidemics may be successfully blocked as was shown by eliminating, e.g., the bluetongue epidemics of ruminants in the years 2006–2009 (Mehlhorn et al. 2007, 2009; Kampen and Werner 2010; Hoffmann et al. 2009). Another example for a blocking of the spreading of an epidemic was successful, when the Chikungunya virus was imported to Central Italy in

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Fig. 2.1 Scanning electron micrograph of the anterior end of an *Ixodes* tick, which is entered into the skin being cut by two saw-like cheliceres

Fig. 2.2 Light micrograph of a female *Anopheles* mosquito sucking sugar solution by help of its two-channeled, injectable piercing mouthparts

Fig. 2.3 Prof. Dr. Heinz Mehlhorn, Düsseldorf University

the year 2007 (Rezza et al. 2007). However, it is clear that strong efforts are always needed to avoid the spreading of bloodsuckers and thus block transmission of agents of diseases. The present book considers the recent situation and shows the endangering situation. However, it does not consider the transmission activities of other bloodsuckers such as leeches (Nehili et al. 1994), bats (Klimpel and Mehlhorn 2013), or fishes (Mehlhorn 2016a, b) (Fig. 2.3).

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Chapter 3
Mosquito Transmission of HIV: Rare or Not Possible?

Diehl Nora

Abstract From its outbreak till today, HIV (human immunodeficiency virus) caused over 35 million dead. If the transmission of the virus would not be restricted to unprotected sexual contact, needle sharing, blood transfusion, and mother to child transmission, this number would probably be tremendously higher. Luckily, HIV has yet not been documented to be transmitted by mosquitoes. Arboviruses (acronym for arthropod-borne viruses)—the viruses that are transmitted by arthropod vectors—are the cause of severe epidemics worldwide. But why is mosquito transmission restricted to certain viruses? This article elucidates the characteristics a virus needs to be spread by mosquitoes and how HIV fits into this picture.

Keywords HIV · Retrovirus · Arbovirus · Mosquito · Vector-based transmission Biological transmission · Mechanical transmission

3.1 Introduction

At the beginning of the 80s, reports of patients suffering from undefined immunological dysfunctions accumulated. The unprecedented and extremely rapid spread indicated a devastating epidemic. Three years later, a retrovirus—subsequently known as HIV—was identified as the cause of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Gallo et al. 1984). Followed by the rapid sequencing of the viral genome (Ratner et al. 1985; Wain-Hobson et al. 1985), ongoing research led to the development of a highly active anti-retroviral therapy (HAART)—a drug cocktail which blocks the virus at different stages of its life cycles. With this lifelong therapy, patients nowadays can survive this former deadly illness. However, rarely one-half of worldwide infected person have access to this therapy. More than 75 million people have been infected with the virus since its outbreak and 35 million people died of AIDS-related illness. 36.7 million people were

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Table 3.1 Characteristics of the HI and dengue virus

	HI virus	Dengue virus
Family	*Retroviridae*	*Flaviviridae*
Genome	+ssRNA, size: 9 kb	+ssRNA, size: 11 kb
Baltimore classification	Group 6	Group 4
Envelope	+	+
Mosquito transmission	−	+
Human to human transmission	+	−
Receptors	CD4, co-receptors: CXCR4 or CCR5	Many candidate molecules, e.g., glycosaminoglycans, lectins
Symptoms	Flu-like illness, followed by an asymptomatic phase	Flu-like illness, rarely: hemorrhagic fever
Prognosis	Lifelong therapy, without therapy: deadly	Recovery within 14 days, very rarely deadly
New infections in 2016	1.8 million	390 million

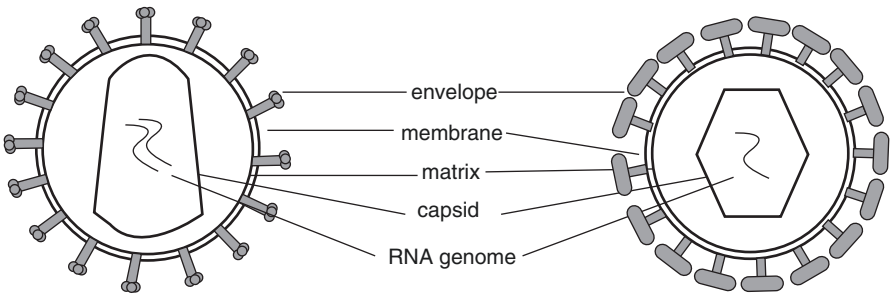

Fig. 3.1 Structure of the HI (left) and dengue virus (right)

living with the virus at the end of 2015 (UNAIDS 2016). Nevertheless, in comparison with the most common mosquito-borne viral disease, the dengue fever, the number of HIV infections seems comparatively small: A recent study estimates that 390 million dengue infections occur annually (Table 3.1) (Bhatt et al. 2013). The dengue fever is caused by the dengue virus, a flavivirus with a positive single-stranded RNA genome (Fig. 3.1), which is mainly vectored by mosquitoes of the genus *Aedes* (principally *Aedes aegypti*). Though not necessarily deadly, this disease can cause hemorrhagic fever, which leads to 22,000 deaths per year. During the last years, the numbers of infections with arthropod-borne diseases increased globally, which is due to increasing mobility, international trade, climatic changes, and approaches to formerly uninhabited areas (Liang et al. 2015). The recent outbreak of the Zika virus, which is likewise a member of the flaviviruses and also transmitted by *Aedes* mosquitoes, in South America, Central America, and the Caribbean, only represents one of several severe threats for human health (Benelli and Mehlhorn 2016).

The lower numbers of people infected with HIV compared to those of, e.g., dengue (Table 3.1) might in part be explained by its different mode of transmission. The main transmission route of the HI virus is via unprotected sexual contact, but also blood, breast milk, and drug needles are origins of infections. However, HIV-containing fluids must directly contact a mucous membrane, damaged tissue, or the bloodstream. Infection via an arthropod vector has not been documented yet. But why are some viruses transmitted by arthropod vectors and other, like HIV, obviously not? This article recapitulates the requirements of a virus to be transmitted by mosquitoes and which of these criteria are accomplished by HIV or not.

3.2 Virus Transmission

Viruses are transmitted by many different routes, while direct transmission between two hosts is the most common way. Thereby, viruses can be embedded within aerosols, body fluids, fecal, or salvia. Depending on the type, they can enter a host orally, intranasally, venereally, or through injured tissue, skin, and mucosa. Another form of virus transmission is mediated by arthropods. This vector-based transmission can either occur mechanically or biologically. In case of mechanical transmission, a vector carries the pathogen on its contaminated mouthparts from one host to another without being infected itself. In case of biological transmission, however, the virus enters the vector to replicate within this host. Biological transmission occurs by far more often than mechanical transmission (Kuno and Chang 2005). As this article focuses on a possible transmission of HIV by mosquitoes, the following passages concentrate on the mechanisms of vector-based and HIV transmission, respectively.

3.2.1 Vector-Based Transmission

As a non-taxonomic clade, arboviruses represent an exceptional group of viruses. Belonging to different taxonomic clusters, the members only share the arthropod-borne mode of transmission. It is commonly accepted that arboviruses primarily originate from arthropod-specific viruses and that this host switch has arisen several independent times during evolution (Halbach et al. 2017). The vast majority of arboviruses are RNA viruses with double-stranded or single-stranded RNA genomes of either positive or negative polarity (Gubler 2001). Members of the taxa alphavirus, flavivirus, bunyavirus, phlebovirus, orbivirus, vesiculovirus, and thogotovirus belong to the group of arboviruses (Weaver and Reisen 2010). In fact, only one arbovirus harboring a DNA genome is known till today: the African swine fever virus. This is probably due to the lower mutation frequency of DNA compared to RNA viruses. DNA viruses simply do not explore the genetic diversity and thus not the possibility to adapt to a new host. Actually, increasing the replication fidelity

and thus minimizing the genetic variability of RNA arboviruses reduces their infectivity in mosquitoes and mice, indicating the absolute need of a certain genetic flexibility (Pfeiffer and Kirkegaard 2005; Coffey et al. 2011).

Importantly, many arboviruses are zoonotic (transmittable from animals to humans), representing a severe danger for public health (Kuno and Chang 2005; Weaver and Reisen 2010). About 300 types of mosquitoes are able to transmit arboviruses, which only represents less than 10% of all mosquitoes living worldwide. Representatives of the species Aedes and Culex transmit the highest number of different viruses. Thus, only a minority of mosquitoes can even serve as a viral vector. Besides the horizontal transmission between two hosts (from vertebrate to vertebrate), arboviruses can also be transmitted vertically to the arthropods' offspring.

The spatial distribution of arboviruses is absolutely connected to the habitat of the arthropod vector, while the temporal distribution depends on the seasons—e.g., arthropods are most active during warm periods. The spread in human populations, on the other hand, depends on several other factors: urbanization, growth of the population, using new ecosystems, increased traveling, climatic changes, and resistance against biocides.

As viruses do not possess genes encoding proteins necessary to execute their own life cycle, they are classified as obligatory intracellular parasites that depend on a host cell to replicate. This genome limitation forces them to adapt perfectly to the host cell conditions (Diehl and Schaal 2013). Replicating in two completely different hosts—like vertebrates and arthropods—however, puts even more adaptive pressure on a virus (Turner et al. 2010; Forrester et al. 2014). But what are the requirements for a virus to be transmitted by arthropods?

In case of biological transmission, the lifecycle of arboviruses within the mosquito begins with the blood meal, whereby they are taken up and initiate the infection in the midgut. They then disseminate to secondary tissue, where further amplification takes place. This is followed by infection of salivary glands and the release of viruses into salivary ducts. When the infected mosquito then sucks blood from a new vertebrate host, a fresh virus generation gets transmitted within the saliva (Hardy et al. 1983). Within the mosquito, the escape from the midgut seems to be a critical bottleneck for many viruses. To leave, they need to infect the epithelial cells (Franz et al. 2015), of which only a few cells seem to be permissive (about 20–30% of cells get infected) even with a high virus dose (Smith et al. 2008). Thus, very high viral titers are required for efficient arbovirus infection.

Besides these tissue barriers in mosquitoes to overcome, arboviruses are faced with the two different immune responses of their arthropod and vertebrate host, respectively. Though arthropods do not possess the powerful immune system and the humoral antiviral response of vertebrates, they also react to an infection and are capable to produce antiviral factors (Fragkoudis et al. 2009; Cheng et al. 2016). However, our knowledge of the insect antiviral response is very poor compared to our knowledge of the vertebrate system. But due to whole genome sequencing, many advances have been made during the last years (Nene et al. 2007; Arensburger et al. 2010).

An essential and best studied antiviral mechanism in arthropods is the RNA interference (RNAi) pathway (Wang et al. 2006; Sanchez-Vargas et al. 2009), though signaling of evolutionary conserved innate immune response pathways like Toll, Imd, and Jak-Stat also exists (Xi et al. 2008; Fragkoudis et al. 2009; Merkling and van Rij 2013). The RNAi pathway senses and cleaves viral RNA to inhibit the virus spread. Most of the studies elucidating the mechanism of RNAi in insects have been performed in the fruit fly *Drosophila* (Wang et al. 2006). Even though the existence of orthologues of core components of the pathway in genomes of mosquitoes indicate conserved structure (Christophides et al. 2002; Waterhouse et al. 2007), we have to keep in mind that *Drosophila* doesn't serve as a viral vector and consequently differences in the molecular biology cannot be excluded.

In principal, the RNAi pathway processes as follows: The infiltrated viral RNA is cleaved into 21-nucleotide-long RNAs by the cellular endonuclease Dicer-2, and the RNA molecules then associate with proteins into an RNA-induced silencing complex (RISC) to guide cleavage of further viral target sequences and thus minimize virus spread (Galiana-Arnoux et al. 2006; van Rij et al. 2006). RNAi is an important antiviral mechanism in arthropods—when RNAi genes are silenced, higher infection rates and severe course of disease are observed (Campbell et al. 2008; Samuel et al. 2016). Beside this well -studied RNAi pathway, recent analyses suggest that the PIWI-interacting RNA (piRNA) pathway also plays a critical role in antiviral strategies as piRNAs are newly synthesized in vector mosquitoes as a response to viral sequences (Miesen et al. 2015, 2016). However, as the pathway was primary only thought to function in genome integrity of germ cells, this illustrates our lack of knowledge about the immune system of arthropods. Though some viral strategies to escape the mosquitoes' immune responses exist (Fragkoudis et al. 2009; Bronkhorst and van Rij 2014), there seems to be a well-balanced trade-off between the immune response and the viral spread (Kang et al. 2008). This is illustrated by the fact that arboviruses do not cause clinical symptoms or influence the behavior or the life span of mosquitoes (Liang et al. 2015; Xiao et al. 2015). Thereby, it is guaranteed that the host remains infectious through its entire life. Suppressing the immune response in *Aedes aegypti* cell cultures or in living mosquitoes results in higher infection rates of the alphavirus Sindbis virus (SINV) and increased mortality (Cirimotich et al. 2009), which would be detrimental to the virus.

On the other hand, vertebrate host can suffer severe diseases, clear the arbovirus infection or die from it. Within the human host, the virus is faced with two defense mechanisms: the innate and the more specialized adaptive immune response. The dengue virus, for instance, is injected from the mosquitoes' salvia into the bloodstream of the vertebrate host. The virus then infects nearby keratinocytes (the most common type of skin cells) and dendritic cells, which then migrate to the lymph nodes (Diamond 2003), where the virus is counteracted by the IFN-dependent innate immune response and later also by neutralizing antibodies. This leads in most cases to the recovery of the patient. The virus, however, has evolved strategies to counteract the immune response in and prolong the infection of its vertebrate host (Morrison et al. 2012). Probably, this is because the virus needs to remain at least as long in the host till it generates titers in the blood high enough to infect new hosts.

The other mode of vector-based transmission is mechanically via contaminated mouthparts of the mosquito. In this scenario, no viral replication in the arthropod takes place. This mode of transmission is quite rare and actually prevalently a veterinary problem (Carn 1996; Chihota et al. 2001; Kuno and Chang 2005). However, at least in laboratory experiments, mechanical transmission has been observed. One prerequisite for mechanical transmission is a high virus titer in the blood, because only small amounts of blood can contaminate the mosquito mouthparts (less than 20 nL) (Hoch et al. 1985). Additionally, the virus must resist the environmental conditions outside the host body like temperature or acidity.

3.3 HIV Transmission

HIV-1 is an enveloped virus with two copies of a positive-sensed single-stranded RNA genome. It belongs to the family of retroviruses (subfamily, *Orthoretrovirinae*; genus, *Lentivirus*), which replicate by using a DNA intermediate (Fig. 3.1). HIV infects cells of the immune system expressing the CD4 receptor on their cell surface. This includes T-cells, macrophages, and dendritic cells (Clapham and McKnight 2001). The attachment to the CD4 receptor leads to conformational changes and exposing of the obligate co-receptor. If the co-receptor is the chemokine receptor CXCR4 or CCR5 is determined by the envelope of the particular HIV strain (Berger et al. 1999). The virus then fuses completely to the host cell membrane to unload the viral genome, which is then translated into double-stranded DNA by the viral transcriptase, which had been incorporated in the viral capsid. The DNA is then imported into the nucleus where it gets integrated in to the host cell genome. This stable integration in to the host genome is the reason, why the infection with HIV persist a lifelong. To produce 18 protein isoforms form only a 9 kb genome, the virus highly relies on pre-mRNA splicing, a process during which intronic sequences are removed and exonic sequences are ligated to build the mature mRNA. Through alternative splicing, which enables differential usage of splice sites, various transcript isoforms originating from one genetic template and thus potentially different proteins are generated (Nilsen and Graveley 2010), enriching the proteomic diversity. To produce the different proteins, HIV completely relies on the cellular spicing machinery (Purcell and Martin 1993). After the translations of these mRNAs into proteins, they get along with two copies of the viral genome enclosed into nascent capsids. Finally the mature virions are released and able to infect further cells.

After initial infection, patients may not suffer from severe symptoms expect flu-like illness. This phase is followed by an asymptomatic stage with an average length of 8 years. Meanwhile, the patients stay infectious. Over time, more and more CD4+ immune cells get irritated by the virus and the constitution of patients' declines. The weakened immune system makes them susceptible for opportunistic infections, which eventually cause their death.

In principal, the transmission of HIV-1 by mosquitoes could take place via the two already descripted ways: biologically or mechanically. These possibilities are discussed below.

3.3.1 *Biological Transmission of HIV by Mosquitoes: Possible?*

For biological transmission by mosquitoes, the respective virus has to successfully replicate within the arthropod host. Having a closer look at the first step during HIV replication, the possibility of a transmission by mosquitoes already becomes highly questionable. As already mentioned, to enter a host cell, the CD4 and either the CXCR4 or CCR5 receptors have to be expressed on the cell surface, which is only true for certain cells of the immune system in higher eukaryotes. Mosquitoes lack cells harboring any of these receptors. As a consequence, absorbed HI viruses cannot enter any cells within the arthropod and thus disappear about 1–2 days after the uptake, which is exactly the amount of time mosquitoes need to digest their blood meal (Bockarie and Paru 1996). In comparison with HIV, arboviruses have a relatively broad cell tropism. Dengue virus, for example, seems able to use many different molecules for the cell entry such as sulfated glycosaminoglycans, lectins, laminin-binding proteins, and glycosphingolipids, both in the vertebrate and arthropod host (Table 3.1) (Hidari and Suzuki 2011).

Imagining that the virus somehow overcomes this hurdle and enters epithelia cells within the arthropods gut, another bunch of barriers is waiting. Several studies showed that hundreds host cell proteins, referred to as dependency factors, are necessary for an efficient HIV replication (Brass et al. 2008; Konig et al. 2008; Zhou et al. 2008; Murali et al. 2011). These proteins are involved in RNA metabolism, protein translation, intracellular transport, or DNA replication. To gain control over the regulation of these cellular processes, virally encoded proteins tackle a spectrum of host cell signaling pathways, which control these activities (Diehl and Schaal 2013). Many of these human proteins involved in HIV replication only have an orthologous gene product with rare similarities in mosquitoes. Considering the genetic differences between humans and *Aedes* species, for instance (*Aedes*, 12,000 genes; humans, 23,000 genes), it becomes obvious that these organisms differ a lot in their molecular biology.

Having a closer look at one essential cellular mechanism for the virus, splicing is only one example of the fine-tuned adaption of the virus to its mammalian host: as already mentioned HIV encodes for 18 protein isoforms, which are all generated from its relatively small 9 kb genome (Jager et al. 2011). To produce several different transcripts ordered and balanced from a single primary transcript, the virus uses extensive alternative splicing (Karn and Stoltzfus 2012). Thereby, not only the specific amount of each mRNA but also its timely expression is critical to the success of the viral life cycle (Klotman et al. 1991; Purcell and Martin 1993). To perform and

coordinate alternative splicing, HIV uses the cellular splicing machinery. This does not only include the host spliceosome, the multi-protein complex that performs the splicing reaction, but also a network of different splicing regulatory proteins. Already minor changes in the amount or the activation status of these proteins are detrimental for the viral replication. Hence, this precisely adjusted system is quite sensitive and unresisting to modifications and perfectly adapted to the human host.

Aside from these molecular requirements of HIV, there are other factors within a mosquito making the life cycle or even the survivor of the virus nearly impossible. As an enveloped virus with a quite instable envelope composed of viral glycoproteins and lipid bilayers taken from the host cell membrane, HIV isn't able to exist for a long time outside a cell (Tjotta et al. 1991; Abdala et al. 1999, 2000). The virus is very sensitive to changes in pH values: below a pH of 5.7, the virus gets destroyed within hours, and also values above 8 are deadly for the virus (Ongradi et al. 1990; Tjotta et al. 1991). Within the mosquito gut pH values between 8.5 and 9.5. were measured (del Pilar Corena et al. 2005), representing a destructive viral environment. Taking together, HIV is not able to enter mosquitoes' cells because of the lack of the respective receptor. Despite that, factors that would be necessary for the viral replication within in the host cell are partially missing in insects. As a consequence, HIV is destroyed in the mosquitoes gut, and thus the biological transmission can technically be excluded.

3.3.2 Mechanical Transmission of HIV by Mosquitoes: Possible?

Having now agreed that biological transmission of HIV by mosquitoes is virtually impossible, what is about mechanical transmission?

Besides the sexual and mother to child transmission, needle sharing among drug users is an increasingly important cause of HIV transmission worldwide. Thereby, contaminated blood in needles, syringes, and paraphernalia are the main sources. During every injection, blood from the user gets inserted into the needle and syringe. If this user is HIV positive and another uninfected drug users utilizes the same paraphernalia without cleaning, the potential virus contaminated blood directly gets injected into the bloodstream, where the virus immediately can infect its target cells without having the hurdle of tissue barriers. Nowadays, people who inject drugs account for 30% of new HIV infections outside the sub-Saharan Africa. Yet, a single incident of shared needle or syringe will not necessarily lead to an HIV infection. The estimation of the infection risk from one injection ranges from 0.6 to 2.4% (Baggaley et al. 2006). The high numbers of newly infected persons who inject drugs can probably be explained with the frequency of contaminated needle usage.

The question is: is there a difference between a needle and a mosquito?

The process of blood sucking by mosquitoes and the injection of drugs with needles highly differ in the mechanism: during blood sucking, mosquitoes send salvia via one tube into the host and suck the host's blood via another tube. The

salvia is composed of substances that prevent blood clotting and platelet aggregation along with vasodilatory substances (Ribeiro and Francischetti 2003). In addition, the salvia contains ant-inflammatory and immunosuppressing proteins, which seem to facilitate viral infections (Edwards et al. 1998; Schneider et al. 2010; Surasombatpattana et al. 2012). Consequently, no blood of the previous host gets injected into the new host. Hence, infection could only appear from blood that glues on the mosquitoes mouthparts. However, this could only be possible for very little amount of blood and thus viral particles. Calculations estimated that more than ten million bites of a mosquito with HIV contaminated mouthparts would be necessary for a HIV-free person to receive a single unit of the virus (Bockarie and Paru 1996).

In sum, mechanical transmission of viruses by arthropod vectors depends on the amount of blood (and the respective virus load) and the way an HIV-free person gets "injected." While shared needles contain considerably higher amounts of blood that directly get injected into the blood stream, potential amounts of HIV-containing blood on mosquito mouthparts can be neglected. Moreover, no blood gets injected into the host during blood sucking.

3.4 Summary

Taken together, we can answer the question asked within the title with a clear "not possible." For biological transmission the virus would need to replicate within the arthropods host, which we have seen can be excluded for the highly specialized HI virus. For mechanical transmission, however, the amount of blood with which the mosquito mouthpart could be contaminated with is by far too low for an infection. In addition, HIV is a quite sensitive virus and thus gets destroyed quite soon outside a host cell. The fact that arthropod-specific viruses are ancestral to arbovirus and that no host change in the other direction has been reported yet makes a mosquito transmission of HIV even more unlikely. Yet, a scenario, in which an arthropod-specific virus, we only don't know, undertakes a host-switch in the future and causes symptoms comparable to HIV, may not be excluded.

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Chapter 4
Dengue: A Silent Killer, a Worldwide Threat

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Abstract Nowadays, the timely and effective management of mosquito-borne diseases is a crucial public health challenge to deal with. Dengue is an emerging mosquito-borne infectious disease transmitted by *Aedes* species. Around 3.9 billion people from more than 128 countries were affected by dengue fever. Recent research outlined that dengue can damage the platelet, disrupting the endothelium bed and provoking immune responses, resulting in severe illness. The illness extends to other vital organs, resulting in homeostasis imbalance. Currently, no vaccines, drugs, or vector control measures showed full efficacy to prevent or manage this arbovirus threat. The present chapter discussed our knowledge about dengue basic

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biology, epidemiology, clinical aspects, and vector control measures. In the final section, several issues to be addressed by further research are critically analyzed.

Keywords *Aedes aegypti* · *Aedes albopictus* · Arbovirus · Culicidae · Epidemiology · Invasive mosquito species · Pesticides · Vaccine

4.1 Introduction

Dengue is a fast spreading mosquito-borne viral disease causing dengue fever (DF) or dengue hemorrhagic fever (DHF), and in severe cases dengue shock syndrome (DSS) (Benelli and Mehlhorn 2016). DHF is a severe febrile disease marked by increased vascular permeability and deformity in homeostasis and may progress to DSS. The life-threatening DSS is a hypovolemic shock that clinically correlated with hemoconcentration. Previous studies highlighted that dengue virus (DENV) replicates in various internal organs, and it has been isolated from the biopsy and necropsy of patients infected by DENV (Póvoa et al. 2014).

4.2 Dengue Virus

4.2.1 Structure

DENV is a member of Flaviviridae family, it consists of 4 serotypes (DENV-1, DENV-2, DENV-3, DENV-4) and can be mostly transmitted to vertebrate hosts by the bites of *Aedes aegypti* and *Aedes albopictus* females (Brady et al. 2012; Sujitha et al. 2015; Benelli and Mehlhorn 2016). During blood ingestion by the vector, DENV from an infected person flows into the midgut of mosquito. The virus then spread to further tissues and starts to replicate. DENV ultimately infect the salivary glands and is inoculated through saliva into new vertebrate hosts during the next mosquito blood meal (Tuiskunen Bäck and Lundkvist 2013). DENV, with a size of 10.6 kb, is a single-stranded RNA; it has a positive-sense polarity, encoding a polyprotein precursor of viral protein of 3411 amino acids long. The RNA is surrounded by a protective coat called nucleocapsid, enveloped by a lipid bilayer and shielded with glycoproteins (Fink et al. 2007).

4.2.2 Replication Cycle and Immune Responses

The basic replication step of flavivirus includes the internalization of the virus particle through endocytosis after attachment to cell surface receptors. Then, in the endosome, the virus fuses with the cellular membrane due to the low pH. This allows the viral particle to dissemble and release its vRNA into host cell cytoplasm. The virus and cellular proteases translate the vRNA into polyprotein, while the NS

proteins make clones of genome RNA. In the endoplasmic reticulum, the C protein and vRNA assemble, forming immature virus particles then transported through the secretory pathway. In the trans-Golgi network, the virus undergoes maturation by the furin-mediated cleavage of premembrane and finally was released from the host cells. During DENV entry into the host cell, the host and viral protease will cleave the precursor generating envelope (Evp), premembrane (prM), and three structural protein capsides (C), which integrate into the mature infective virus particle. Envelope proteins are functioning at the viral entrance into the host cell, as well as the antiviral target for enhancing and neutralizing antibodies (Fink et al. 2007; Kurane et al. 1995; Murphy and Whitehead 2011; Whitehorn and Farrar 2010). The size of E glycoprotein is approximately 55 kDa, and it owns three domains (1, 2, and 3), at which the genes are responsible for membrane fusion, dimerization of the E protein, cell receptor binding, and cross-reactive epitopes (Lai et al. 2008). The E glycoprotein DENV virion is the major exposed outer protein that mediates immune response via neutralizing antibodies. It works by binding cell receptors, fusing with host cell membranes in the process of viral penetration, then mediating viral assembly and rupture, and budding off, hence displaying antigenic determinants that elicit immune responses (Fig. 4.1). It is the amino acid sequence of E region in the structure of DENV that differentiates and defines the unique four serovars (DENV-1, DENV-2, DENV-3 and DENV-4) (Lanciotti et al. 1997). Besides, DENV also contains seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4A, NS4B, and NS5) involved in replication and assembly of the virus (Fig. 4.1) (Fink et al. 2007; Kurane et al. 1995; Murphy and Whitehead 2011; Whitehorn and Farrar 2010) (Fig. 4.2).

4.3 Epidemiology

The first recorded evidence of dengue was traced back as early as 992 A.D. in China, as the signs of dengue-like disease back then were similar to those of dengue. DF started to make the appearance in seventh century but with low frequencies. Before

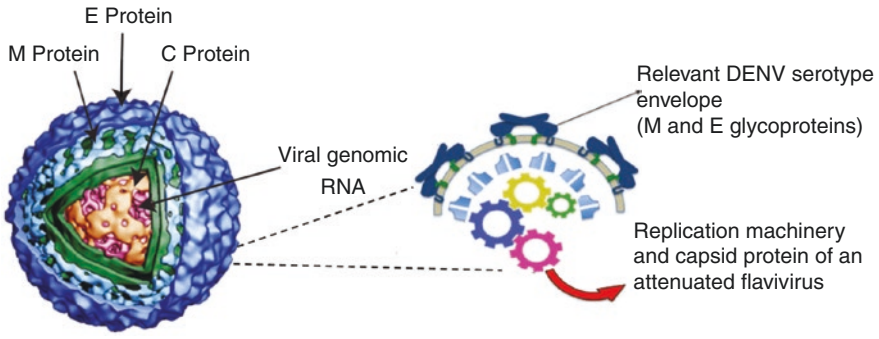

Fig. 4.1 Structure of dengue virus (Adapted and modified, source: de Angel and del Valle 2013)

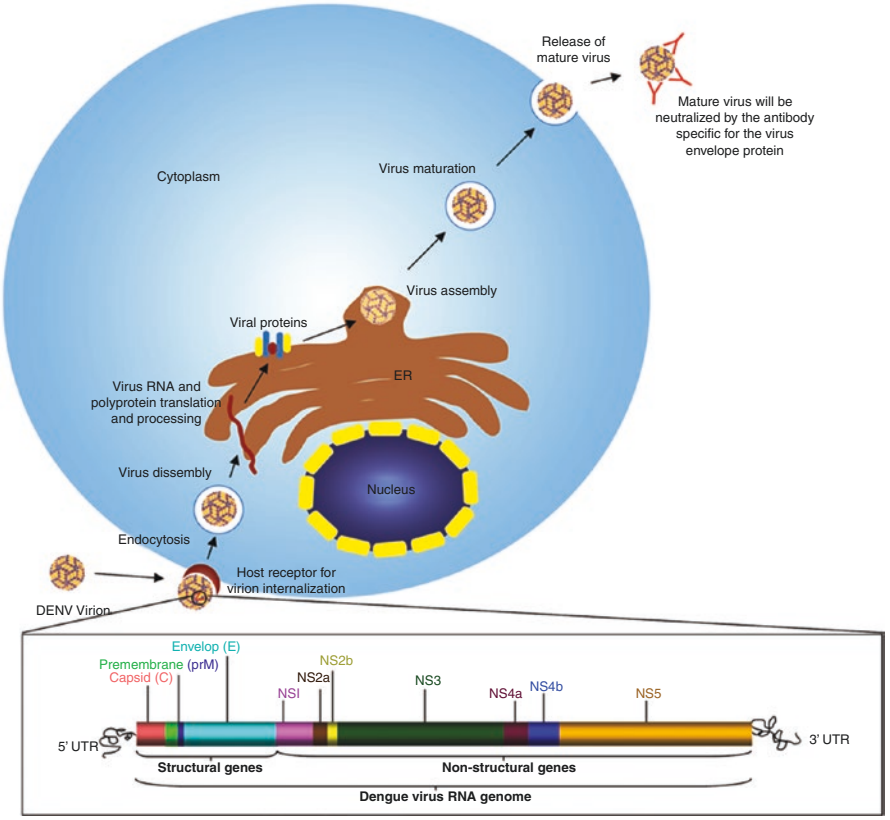

Fig. 4.2 Dengue virus replication cycle and immune response development in the host. The structural genome of DENV is given in the lower image (Adapted and modified, source: Rothman 2011)

1970, roughly ten nations were affected by severe dengue occurrences (Gubler 2002, 2005; Gubler and Meltzer 1999). However, the presence of dengue has experienced major changes around the world in the past few years. Dengue illness now is common in more than 128 nations with the approximation of 3.9 billion people in tropical and subtropical region are at great threat of DV infection (Murray et al. 2013; Benelli and Mehlhorn 2016). At the beginning of this century, the disease arose in Bangladesh, Bhutan, Nepal, the Galapagos Islands (Ecuador), Timor-Leste, Easter Island (Chile), and Hawaii (USA) (Dorji et al. 2009; Ahmed 2003; Bharaj et al. 2008). In 2012, WHO argued that main dengue-risk countries were Bangladesh, Bhutan, Cambodia, Malaysia, Hong Kong, India, Pakistan, Indonesia, Nepal, Vietnam, Thailand, the Philippines, and the America. Dengue cases are growing as the disease spreads to new areas, and explosive outbreaks are occurring as well (Benelli 2015). Dengue fever is currently a major public health concern in the present decades, where it affects more than 50 million people every year, in over 100 countries (Teo et al. 2009; World Health Organization 2009). Since from last 50 years, the World Health

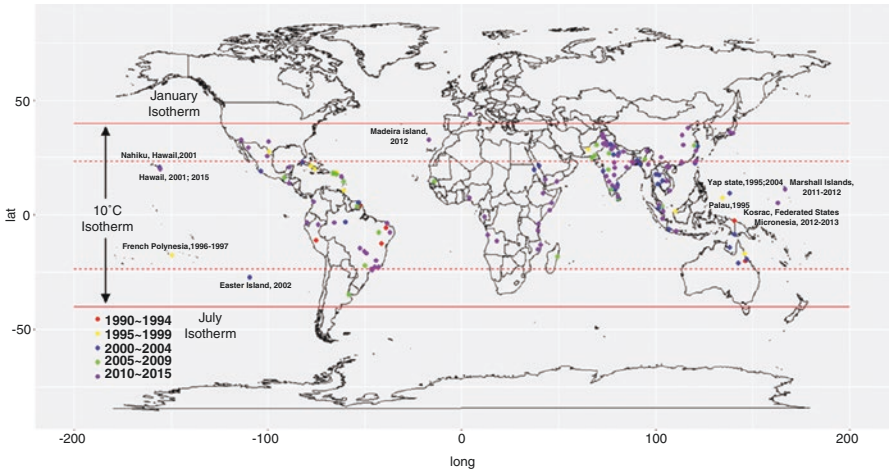

Fig. 4.3 Distribution of dengue outbreaks from 1990 to 2015 (adapted and modified, source: Guo et al. 2017)

Organization has records stating that the prevalence of DENV infection has raised to 30-fold with growing geographic extension to new countries (World Health Organization 2009). Other than that, Brady et al. (2012) in their recent study state that DENV causes more than 390 billion infections every year. Dengue not only is causing threat to the people health but also to the economy of people and nations (Fig. 4.3).

4.4 Dengue Virus Infection in Humans

4.4.1 Clinical Manifestation

Dengue viral infection has been characterized into several categories according to clinical symptoms. Undifferentiated febrile illness (UF) usually appears with maculopapular rash, which cannot be diagnosed clinically. DF is characterized by a milder disease with several clinical symptoms, including high fever for 2–7 days (>39 °C), severe headache, myalgia, arthralgia, nausea, rash, vomiting, pain behind eyes, muscle, and joint or bone pain. Leukopenia, thrombocytopenia, and bleeding may also be observed. In life-threatening condition DENV causes DHF, which results in hypotension, marked thrombocytopenia, hemoconcentration, plasma leakage, leucopenia, hemorrhage, hepatomegaly, hypoproteinemia, circulatory failure, and mortality in 1–5% of infected individuals (Teo et al. 2009; Sharma et al. 2011). Dengue shock syndrome (DSS) exhibits similar symptoms as DHF. However, the patient will experience shock, due to fall of body temperature, circulatory failure, and circumoral cyanosis, which may progress to death if proper volume replacement treatment does not take place (World Health Organization 2009) (Fig. 4.4).

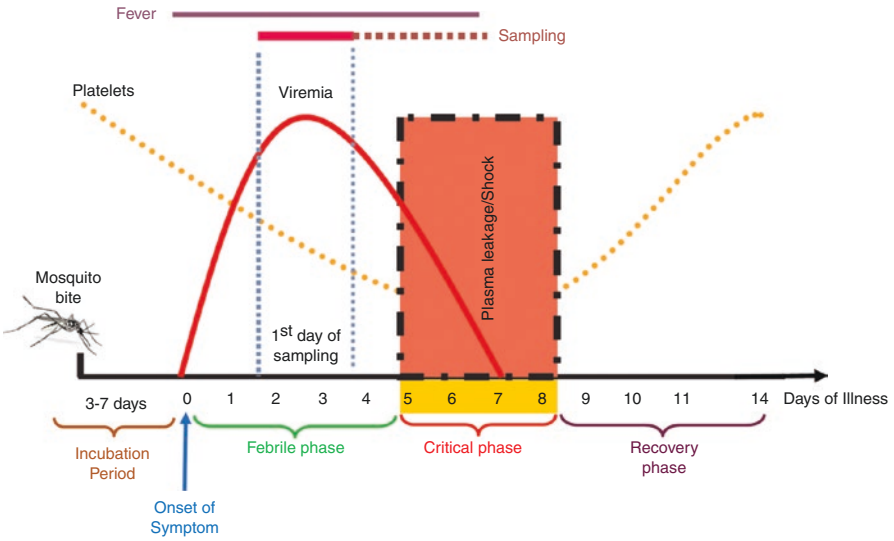

Fig. 4.4 Chronology of phenomenon in dengue infection. DENV vectored by mosquito biting activity causes clinical symptoms, such as fever, approximately from 3rd to 7th day of incubation. At the beginning of fever, viremia can be detected and can last up to 5–7 days. Usually patients only seek for help after 2–3 days of fever, which correspond to the peak of viremia. Severe condition of dengue, DHF and DSS often occur during or as soon after viral clearance and during fever reduction. The lowest level in platelet counts are commonly noticed during plasma leakage or 5 days after fever (adapted and modified, source: Tsai et al. 2011)

4.4.2 Type of Organs Affected

Platelets, bone marrow, endothelial cells (ECs) of the blood vessels, and the liver are among the organs that are severely affected and involved in DENV infection, which will be further discussed below.

4.4.2.1 Dengue Virus Infecting Platelet and Bone Marrow

Studies by Kapur and Semple (2017) and Von Hundelshausen and Weber (2007) illustrate that platelets can significantly contribute to immune response, where they can bind with lymphocytes (Li 2008) and encounter the infectious agents (Elzey et al. 2003). The mechanism that expedite to thrombocytopenia includes decreased platelet production, increased platelet consumption, or immune complex which lead to platelet lysis (Oishi et al. 2007; Srichaikul and Nimmannitya 2000). Since bone marrow is the place for blood cell production, its suppression within 2–4 days by DENV infection causes deficiency of blood cells leading to thrombocytopenia, leukopenia, and anemia (De Azeredo et al. 2015).

A previous bone marrow study reveals that DENV infection causes the process of megakaryopoiesis to be declined (La Russa and Innis 1995). A study by de Araújo et al. (2009) prove that DV targets the bone marrow and also the hematopoietic system, by isolating viral RNA from the marrow specimen of infected patient. Other than that, a previous study found that the human cord blood progenitor's cell proliferation is halted by DV infection, while another study reported that the differentiation of CD34+ progenitor cells into megakaryocyte is arrested by DV2 infection through apoptosis of infected cells (Basu et al. 2008; Murgue et al. 1997). These investigations give strong evidence that the DENV, indeed, infect the hematopoietic progenitors and finally halt the megakaryopoiesis and thrombopoiesis. Hematopoiesis can also be arrested by DENV by infecting the stromal cell, which is proven by Pascutti et al. (2016). They found that the infected bone marrow cells express DENV antigen after long-term marrow cultures. In addition to that, a study by La Russa and Innis (1995) suggests that the cytokines produced by infected stromal cells were different from the ones from uninfected stromal cells. This condition may also be one of the mechanisms leading to marrow suppression during the dengue infection (La Russa and Innis 1995).

Thrombocytopenia and platelets dysfunction are the major features in DENV infection. This condition may occur from 4 to 7 days after the onset of fever (Srichaikul and Nimmannitya 2000). DENV not only have been found inside polymorphonuclear leukocytes, monocyte, macrophages, and dendritic cells but also in megakaryocyte progenitors and circulating platelets (Oishi et al. 2007; Noisakran et al. 2010; Onlamoon et al. 2010; Shinji 1989). DENV has been shown to lower the circulating platelet counts by its attachment or entry into platelets or their precursors. It is well known that platelets have the ability to engulf foreign particles, such as latex beads, bacteria, and viruses, including blood-borne pathogens (Youssefian et al. 2002). In DENV infection cases, it generates antiplatelet antibodies and causes destruction of platelets. Bozza et al. (2008) suggest that immune complex-mediated platelet destruction is an important factor in dengue individual that lead to thrombocytopenia. Anti-DV antibodies generated by host immune system can cross-react with platelets and lead to platelet clearance (Lin et al. 2006), and this autoimmune hypothesis is supported by several research studies. For example, Lin et al. (2006) observed that the antiplatelet IgM level is higher in severe DENV infections compared to classical DF. In clinical setting of acute phase of secondary DENV infection, there is increase amount of immunoglobulin (PAIgM/PAIgG), which is associated with platelet and also increased the platelet phagocytosis by the macrophages, in parallel with thrombocytopenia (Saito et al. 2004; Tsai et al. 2012).

Besides, complement-mediated platelet lysis may be also induced by the presence of antinonstructural protein (NS1) antibody in DENV-infected patient serum (Noisakran et al. 2010; Zucker-Franklin et al. 1990). NS1 antibody with the productive DV infection cross-reacts and activates the endothelial cells. Infected endothelial cells express high E-selectin, which enhance the adherence of platelet (Huang et al. 2000). This reaction leads platelet to express P-selectin, which in turn interacts

with leukocytes (platelet-monocyte and platelet-neutrophil aggregation) (Dalrymple and Mackow 2011; Ghosh et al. 2008; Krishnamurti et al. 2002). The endothelial cell-platelet-leukocyte interaction may be beneficial for dengue pathogenesis and thrombocytopenia (Noisakran et al. 2010). Other than that, it has been shown that the abnormal activation and inhibition of platelet aggregation is facilitated by the dengue individual serum (Lai et al. 2008; Li 2008). Recent studies conclude that DV also directly interact and activate platelets inducing morphological changes (Basu et al. 2008).

4.4.2.2 Dengue Virus Infection on Endothelial Cells

In addition to the platelets and bone marrow, DENV can also cause plasma leakage, the most characteristic features and best indicator of disease severity in DHF or DSS by infecting the ECs of blood vessels. Plasma leakage is manifested by the combination of hemoconcentration, ascites, or pleural effusion (World Health Organization 2009). These conditions become obvious on days 3–7 of illness, at which the DF is resolved. Plasma leakage is caused by the elevated fluid diffusion in vessel permeability, occurs systemically, and progresses quickly. However, if the patient receives prompt fluid resuscitation, plasma leakage may resolve within 1–2 days (Srichaikul and Nimmannitya 2000; Bhamarapravati 1989; Bhamarapravati et al. 1967). Plasma leakage was also characterized by a headache, biphasic fever, rash, myalgia, pain in diverse body parts, leukopenia, and lymphadenopathy (Bhamarapravati et al. 1967; Henchal and Putnak 1990).

Previously, plasma leakage was thought to occur in DENV-infected patients due to amended vascular permeability instead of structural demolition of ECs. However, it was found that the release of cytokine or mediator in DENV infection becomes the probable cause of functional alteration in ECs. A previous study by Huang et al. (2000) found that the DENV is capable of infecting human ECs in vitro and results in the production of diverse chemokines and cytokines including IL-8, IL-6, and RANTES. This study suggests that the ECs can be a target for DENV infection and may contribute to the pathogenesis of DHF (Huang et al. 2000).

The possible mechanism that contributes to plasma leakage increase is the microvascular permeability (Bielefeldt-Ohmann 1997; Biron et al. 1999). Overreaction of the immune response after DENV infection leads to the tsunami production of cytokines, which affect hepatocytes, monocytes, and ECs, in addition to virus clearance. Moreover, the reaction also produces an abnormal amount of autoantibodies to platelet and ECs, which eventually cause the functional damage of these cells (De Azeredo et al. 2015). The secretion of chemokines and cytokines by the immune-mediated responses or by the infected ECs to the infection has also been pointed out by several previous studies (Cardosa 1998; Citarella et al. 1997). Halstead and O'Rourke (1977) suggested that these chemokines and cytokines affect the gap junction of the ECs, resulting in a momentary increase in the endothelium permeability. A study by Chen et al. (2007) found that the high titers of DENV inoculation by intradermal predispose the ECs to TNF-α-induced cell death, which eventually result in endothelium damage and hemorrhage (Fig. 4.5).

Endothelial cells (ECs) infected with DENV are also capable of initiating complement and generating the adhesion molecules expression such as ICAM-1 (Nielsen 2009). When ICAM-1 is expressed, the polymorphonucleated (PMN) cells and mononucleated cells increase, respectively, with the release of RANTES IL-8 and chemokines, resulting in elevated thrombomodulin release and vasopermeability, a marker of EC impairment. This is supported by a previous study of Cardier et al. (2006), where they scrutinized levels of soluble vascular cell adhesion molecule and intercellular adhesion molecule (sVCAM-1 and sICAM-1), and the presence of circulating endothelial cells (CECs) in the peripheral blood of DHF patients are the evidence of vascular damage, which become an evidence of endothelium damage and activation in DHF patients.

In the absence of complement, increased vasopermeability and thrombomodulin is also released in ECs by co-incubating the DENV-infected ECs with anti-DV antibodies. EC structural damage occurring in vivo results from increased levels of circulating thrombomodulin in the acute stage of DHF/DSS (Basu et al. 2008). It is found that immune-mediated damages, direct viral cytopathic effects, and anti-DENV antibodies can lead to structural impairment to infected ECs. Because the endothelium plays an essential role in retaining hemostasis, infected ECs may distort the anticoagulant or procoagulant balance of endothelium and escalate the tendency of bleeding. Meanwhile, the action of platelet by the activated ECs may also induce the progression of thrombocytopenia (Assinger 2014) (Fig. 4.5).

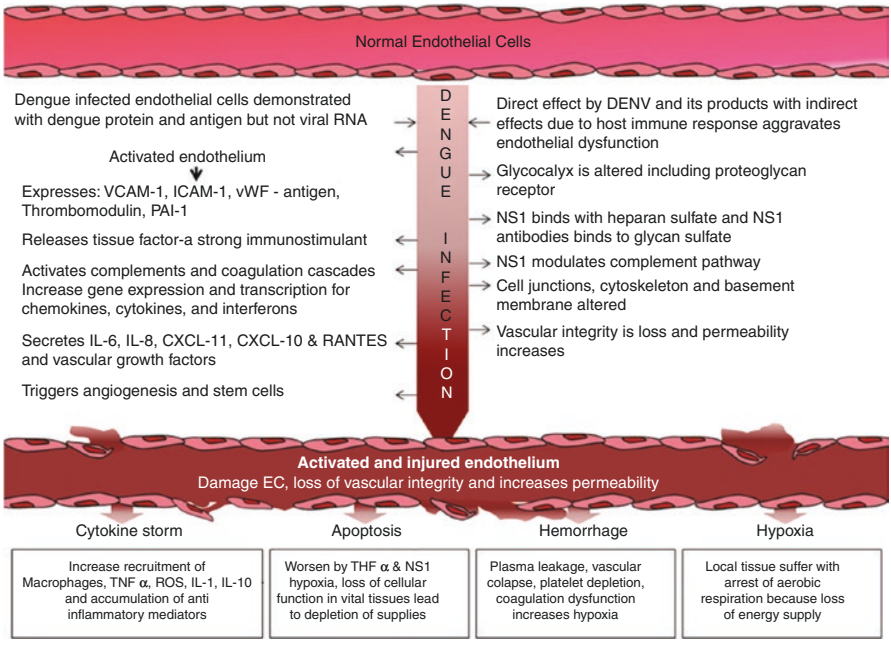

Fig. 4.5 Effect of dengue virus infection on endothelial cells and blood vessel (adapted and modified, source: Priya et al. 2017)

4.4.2.3 Dengue Virus Infection on Liver

DENV infection also causes the impairment of various organs, especially the liver (World Health Organization 2009). The liver is the largest internal organ, and is responsible for many cellular requirements for the whole body. It has endocrine and exocrine functions and includes hepatocytes, Kupffer cell (KCs), Ito cells, ECs, and cholangiocytes. Hepatocytes are key cells involved in the synthesis of liver molecules; liver secretes bile and plasma proteins, detoxifies harmful chemicals, and stores glucose, glycogen, vitamins, and minerals, like iron. The KCs are specialized macrophages found in the sinusoidal lining. KCs engulf aged cells and store proteins and minerals for reuse. They also engulf bacteria and debris in portal circulation and function as antigen-presenting cells. Endothelial cells (ECs) are present in various locations, like lining the sinusoids and blood vessels, including the portal vein. Ito cells are mesenchymal cells that are present in perisinusoidal spaces and store fat-soluble vitamins (Sato et al. 2003). The liver plays a major role in mammals. It is the frontier between the blood and digestive system, where it processes the nutrients absorbed by the digestive tract and supplies them to other regions of the body. Additionally, the liver has an ideal position in the circulatory system, where it assembles, transforms, and accumulates all metabolites, neutralizes the circulatory system, and eliminates toxic materials from the blood. The liver also generates various plasma proteins, such as carrier proteins, fibrinogens, and albumins.

Being damage to the liver, dengue also causes further damage to other sites of the body. Previous studies conducted on humans and other animals have suggested that the DENV-infected liver has several functional abnormalities, viral antigen presentation, and tissue injury. Several human studies have found that a common characteristic of DENV-infected livers is the small foci of necrosis and microvesicular steatosis (Couvelard et al. 1999). However, these studies are limited. The majority of human dengue infection studies are based on hematological and biological characteristics, since it is not easy to access tissue samples from living patients (An et al. 1999; WHO Initiative for Vaccine Research and World Health Organization, Department of Immunization Vaccines and Biologicals 2008; Halstead 2007; Kalayanarooj et al. 1997; Lin et al. 1998; Rosen et al. 1999). Thus, serum liver enzymes (i.e., alanine and aspartate aminotransaminase) have been used as indicators to detect the involvement and severity of liver damages caused by dengue infections. During DENV infections, the inflammatory process leads to a parenchymatous lesion that releases these enzymes into the blood. The increase of aminotransaminase can be detected in the acute phase of the disease and subsequently reduced as the liver recovers. The ALT was mainly present in liver cells and present in a very low concentration in other tissues, while AST were also found in other tissues such as cardiac and muscles. Elevated aminotransaminase is found in case of cirrhosis, hepatocellular carcinoma, biliary obstruction, and infectious diseases including dengue (de Souza et al. 2007). Various human and animal studies have reported the elevated level of AST and ALT during DENV infection. Dengue patient reports by Lee et al. (2012) from Singapore found that 86 and 46% out of 690 patients have an elevation of AST and ALT, respectively.

An increasing number of studies showed that DENV usually targets Kupffer cells as well as hepatocytes of the liver by the attachment to the compatible receptors on liver cells (Marianneau et al. 1999; Seneviratne et al. 2006; Thongtan et al. 2004). In in vitro and in vivo research, it has been proved that DENV infection cause apoptosis to the liver cells (Couvelard et al. 1999). These pathological changes are not limited to the DENV infection alone but can be also due to diverse pathways involved such as hypoxic mitochondrial dysfunction, immune responses, and endoplasmic reticular stress (Matsuda et al. 2005; Thongtan et al. 2004). DENV infection also leads to release of cytokine storms such as interleukins IL-10, IL-5, IL-6, IL-4, and IL-2. In addition to that, tumor necrosis factor TNF-α and interferon IFN-γ are also released in response to dengue infection (Chaturvedi et al. 1999). As mentioned above, the immune reaction enhancement and cytokine storm causes severe liver diseases during DENV infections. During recurrent infection, there are various interacting factors, i.e., the virus, host condition, and antibody-dependent enhancement, which eventually lead to DHF and DSS (Halstead and O'Rourke 1977). Since the liver is the most important organ in the body to maintain the physiological process, DENV infection of the liver may lead to further damage to other sites of the body, and this disables the body mechanism to recover to a normal state. Figure 4.6 provides a detailed flow chart of the pathological process resulting in liver cell damage.

4.5 Current Measures to Manage Dengue Infection and Vector Populations

Currently, the only convenient and feasible treatment for dengue infection is supportive fluid resuscitation to resolve plasma leakage, oral rehydration, and platelet or blood transfusion to resolve thrombocytopenia (World Health Organization 2009). With no effective dengue drugs and vaccines, controlling and preventing DF outbreaks becomes a crucial measure for keeping people safe. Global alliance of international health agencies has advised the community to take immediate personal action in order to control the spread of the dengue vectors (Achee et al. 2015).

Current vector control measures include environmental management and biological or chemical approaches. To reduce the spread of dengue virus, vector reduction strategy has been done reducing the occurrence of mosquito breeding sites, with special reference to urban environments. (Benelli and Mehlhorn 2016). By doing so, *Aedes* females are prevented from laying their eggs. Installing water systems to replace water-storage containers, educational approaches to teach communities about the risks of mosquito vectors, as well as the usage of biocontrol agents and biotechnological tools including GM mosquitoes, adult repellents, and ovitraps are other examples of attempted control strategies (Benelli et al. 2016; Wilke et al. 2018). None of them showed conclusive results as standing-alone control tools, therefore, their employ under an Integrated Vector Management scenario, post-evaluation by the WCAG of WHO is required (Benelli and Beier 2017). Chemical control

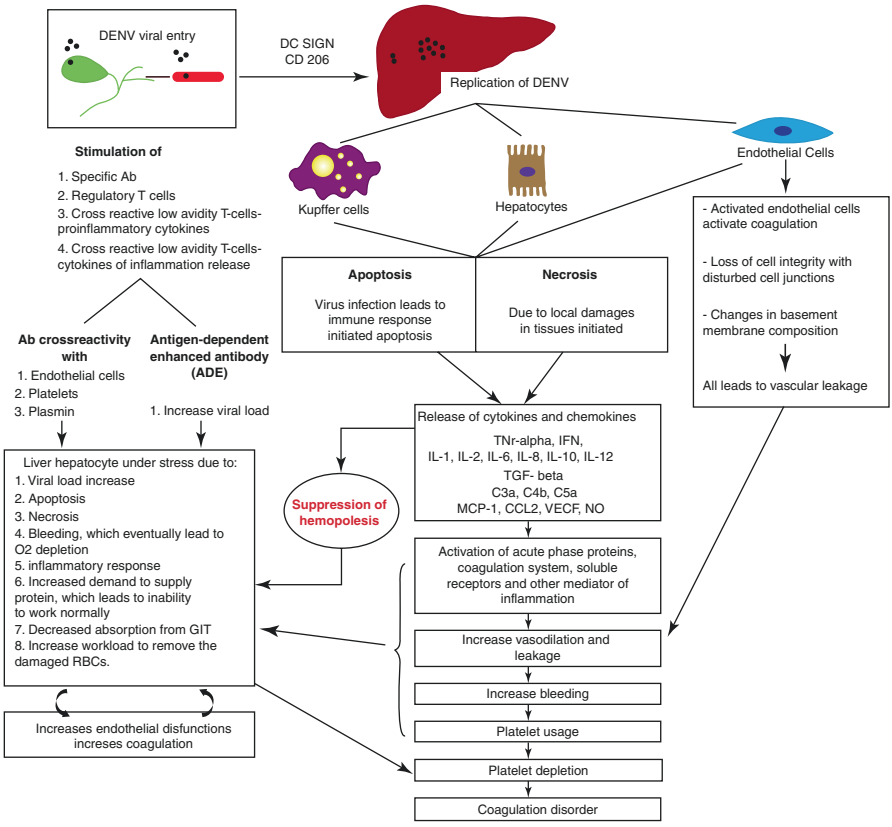

Fig. 4.6 Cumulative pathological process resulting in liver cell damage. DENV viral entry in liver and its consequences are briefly described in the flow chart (adapted and modified, source: Sakinah et al. 2017)

approaches include insecticides and bio- and green insecticides to kill mosquito larvae, adult mosquitoes, and even juvenile hormone analogues to arrest the transformation of young instars into adults. However, cautions are necessary as most pesticides currently employed are toxic to humans and other species (Benelli et al. 2018).

The progress of various investigations has led to the discovery of potential anti-dengue drugs and vaccines. However, still, there are no promising candidates. Mahalingam et al. (2013) recently reported that selected vaccines have been found to be ineffective against dengue infection. There are many reasons for the ineffectiveness of the anti-dengue drugs, including the presence of various serotypes complicating the selection of potential drug, the late occurrence of antiviral therapy activity, and the side effects of drug consumption. Even though the antiviral drug would help in viral clearance, its efficacy in the recovery of damaged organs caused by DENV infection is still questionable. Apart from that, its expensive cost may also halt its usage in underdeveloped nations (Halstead 2016; Harapan et al. 2017).

4.6 Which Future for Dengue?

A wide number of experts states that there will be a worldwide increase of DENV cases, as reported also reporting by WHO (Gubler 2005; Åström et al. 2012). Climate changes and virus adaptation are possible factors that may increase future dengue infections. It is well known that temperature changes play a key role for virus replication, vector survival and infection (Gubler 2002, 2005; Patz and Reisen 2001; Reiter 2001). The Intergovernmental Panel on Climate Change has predicted that global temperature will rise in future. This condition has become a major concern as the rise in temperature might expand the mosquito vector survival in addition to its migration to currently non-endemic region (IPCC 2007; Benelli 2018). Viral adaptation to naïve host can be observed when the envelop protein modifies in order to correspond with the epidemic or endemic emergence (Wang et al. 2000). The future is worrisome as DENV infection will cause extensive negative impact not only to the individual health but also countries' expenses for the treatments.

Overall, our analysis pointed out that current control measures does not stop the spread of DENV infection, while anti-DENV drugs and vaccines are not a promising candidate to treat the patients. As moving with the progression in medical field, treating DENV with stem cells would be ideal. On the other hand, possible ways to treat the DENV infection with stem cells need to be study thoroughly to avoid misleading consequences on patients' recoveries.

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this study.

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Chapter 5
Vector Potential of Mosquito Species (Diptera: Culicidae) Occurring in Central Europe

Helge Kampen and Doreen Walther

Abstract After the eradication of endemic malaria in the mid-twentieth century, research on native mosquito species was neglected in Europe for decades. With no evidence for the transmission of life-threatening pathogens, mosquitoes were not considered important vectors anymore. Public, political and scientific interest in them as vectors of disease agents has only increased again with the advent of invasive species and putatively exotic pathogens such as dengue and chikungunya viruses, as a consequence of continuing globalisation. While there is quite useful data on the vector competences of invasive mosquito species, which—due to their involvement in the transmission of disease agents in other parts of the world—had been in the focus of research already before their introduction into Europe, little knowledge exists on the vector potentials of indigenous mosquito species other than *Anopheles* species able to transmit malaria parasites. Only recently, the screening of field-collected mosquitoes for pathogens has been intensified in Europe, but findings usually remained unclear regarding whether the pathogens had just been ingested during blood-feeding or had really been able to infect their insect host and continue their developmental cycle in order to be transmitted during the next blood meal. Likewise, studies are largely lacking investigating the transmission of pathogens, either endemic or exotic, by European mosquito species in the laboratory, which is the ultimate proof of their vector competence. The present contribution compiles literature data on demonstrations of pathogens in field-collected specimens of mosquito species occurring in Central Europe, although not necessarily collected in Central Europe, as well as of laboratory infection studies with mosquito species occurring in Central Europe. The literature overview shows that mosquito vector research on indigenous species has to be further intensified in order to prepare well-founded risk assessments of outbreaks of mosquito-borne diseases of humans and animals in Central Europe.

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5.1 Introduction

Owing to recent outbreaks of mosquito-borne diseases, such as dengue, yellow fever, chikungunya and Zika, the dipteran family Culicidae has globally regained tremendous public, political and scientific attention. In fact, together with ticks, the culicids include the most important arthropod vectors worldwide in terms of numbers of pathogens transmitted and morbidity and mortality caused by these.

While Europe was relatively safe from mosquito-borne diseases during the last few decades, these are currently emerging and resurging together with old and new vector species. This trend can be observed globally: supported by technological progress and global trade and travel, both mosquitoes and the pathogens they transmit are increasingly often introduced into regions of the world remote from their native distribution areas (Tatem et al. 2006). Climate change might support their establishment. Given this development, there is not only reason for concern about newly introduced vectors transmitting new pathogens in new areas of the world, but endemic species might turn out to be efficient vectors of the new pathogens, and endemic pathogens might be much more efficiently transmitted by imported vector species.

Interestingly, much more is known about the vector competences of so called exotic or invasive mosquito species than of those endemic to Central Europe. The first group has usually made an appearance as vectors of disease agents either in their native distribution areas or in other areas of the world where they had been introduced and has therefore been subjected to vector competence studies already for the purpose of risk analyses. By contrast, most of the mosquito species occurring in Central Europe have hardly ever played noteworthy roles in the transmission of pathogens on this continent. Few of them, mainly those which are also native to other parts of the world, have been found to be carriers of pathogens in the field or demonstrated to be vectors in the laboratory. With adequate ecological conditions provided, they can possibly act as natural vectors in Central Europe in the presence of infection sources.

Both climate change and globalisation considerably increase the risk of mosquito-borne disease transmission in Central Europe in that conditions for the development of both vectors and pathogens generally improve (e.g. warmer temperatures accelerate the extrinsic pathogen incubation period and boost mosquito biting rates and population growth), and infection sources (e.g. travellers returning from tropical countries) become more frequent.

This contribution will compile important viruses and other pathogens potentially transmitted by mosquito species, either native or invasive, occurring in Central Europe. The compilation includes field and laboratory findings.

5.2 Mosquito-Borne Diseases in Europe Now and Then

Mosquito-borne diseases are certainly not confined to the tropics. They are rather linked to the presence of vector-competent mosquito species and may occur in regions where temperatures allow for the completion of the extrinsic development of the pathogens. Thus, Europe was not excluded from suffering from mosquito-borne diseases until the twentieth century. Endemic malaria had been described in Greek poetry already hundreds of years BC and was eliminated from Europe only in the 1970s (Bruce-Chwatt and de Zulueta 1980). Even yellow fever and dengue were not uncommon and broke out until the early twentieth century (Morillon et al. 2002; Schaffner and Mathis 2014).

The occurrence of malaria was obligatorily correlated with the distribution of certain *Anopheles* species (Fantini 1994) which have always belonged to the indigenous European mosquito fauna. These were extensively controlled during the European antimalaria campaigns of the twentieth century but have recovered and are again widely distributed (Ramsdale and Snow 2000). By contrast, the former European malaria parasite strains have become extinct, but autochthonous malaria cases and episodes caused by local transmission of imported tropical parasites occasionally occur (e.g. Krüger et al. 2001; Danis et al. 2013).

Yellow fever and dengue were linked to *Ae. aegypti*, a thermophilic mosquito species not native to Europe. However, it was regularly introduced by ships returning from tropical countries and succeeded in building up local populations at least during the warm seasons. For uncertain reasons, this species disappeared from Europe by the middle of the last century (Holstein 1967).

There are some mosquito-borne viruses that were detected in Europe decades ago and have since been found to circulate. These are therefore considered endemic: Sindbis, Batai, Tahyna, Inkoo and snowshoe hare viruses, which are of minor pathogenicity to humans and other vertebrates, and Lednice virus, which is thought to be non-pathogenic (Hubálek 2008).

The only bacterial disease associated with mosquito bites is tularaemia. Detections of its etiologic agent, *Francisella tularensis*, have been made in various mosquito species (Hopla 1974), although mechanical rather than biological transmission appears to occur. In Fennoscandia, thousands of cases of tularaemia have been diagnosed since the 1930s (Tarnvik et al. 1996), with infections mainly occurring during the seasonal peak of mosquito activity in late summer and early autumn (Eliasson and Bäck 2007).

Some other mosquito-borne diseases must be regarded emerging and resurging in Europe: West Nile fever, chikungunya, dengue, Usutu and dirofilariasis.

West Nile virus was detected for the first time already in the early 1960s (Hannoun et al. 1964), but disease outbreaks affecting horses and humans became more frequent and severe only recently (Hubálek and Halouzka 1999; Sambri et al. 2013). For uncertain reasons, cases remained restricted to the southern parts of Europe

although there is serologic evidence for the virus to circulate in more northern parts (e.g. Buckley et al. 2003). Chikungunya broke out for the first time in continental Europe in northern Italy in 2007, with further cases subsequently occurring in France, Italy and Spain (Tomasello and Schlagenhauf 2013; Delisle et al. 2015; Calba et al. 2017; Venturi et al. 2017). A similar situation was given with dengue which, after decades of absence from continental Europe, re-emerged in 2010 in Croatia and France and afterwards sporadically reappeared in France (Tomasello and Schlagenhauf 2013). Usutu fever seems to be a primarily avian disease although severe cases have been described in humans recently (Santini et al. 2015; Simonin et al. 2018). Usutu virus was first detected outside Africa in 2001 when it caused conspicuously high mortality among blackbirds around Vienna (Weissenböck et al. 2002). Later findings suggested that the virus had long before been present in Europe unnoticed (Weissenböck et al. 2013). After the 2001 outbreak, the virus was found in other European countries both in mosquitoes and dead birds (Ashraf et al. 2015).

Dirofilariasis is of growing concern in Central and eastern Europe. Numbers of canid and human cases of both *Dirofilaria immitis* and *D. repens* infections have continuously been on the rise for years in Italy and other endemic southern European countries in the Mediterranean, but due to spreading northwards, the worms are now increasingly often registered, both in vertebrates and in mosquitoes, in European areas previously regarded non-endemic (Genchi et al. 2011; Morchón et al. 2012; Sassnau et al. 2014).

5.3 Mosquito Species in Central Europe

The Central European region dealt with in this contribution comprises Austria, Belgium, the Czech Republic, Germany, Hungary, Liechtenstein, Luxembourg, the Netherlands, Poland, Slovakia and Switzerland (Fig. 5.1). Mosquito inventories exist for Austria (Mohrig and Car 2002), Belgium (Boukraa et al. 2015), Germany (Becker et al. 2011), Hungary (Tóth and Kenyeres 2012), Luxembourg (Beck et al. 2003), the Netherlands (Ibáñez-Justicia et al. 2015), Poland (Kubica-Biernat 1999), Slovakia (Jalili et al. 2000) and Switzerland (Briegel 1973), although in some cases probably not up-to-date considering the recent introduction and spread of invasive species in Europe. In Germany alone, nine new species have been described since 2007 (Kampen et al. 2017), with five of them (*Ae. albopictus, Ae. japonicus, Cs. longiareolata, Ae. koreicus, An. petragnani*) being invasive (Pluskota et al. 2008; Schaffner et al. 2009; Kampen et al. 2013b; Becker et al. 2016; Werner et al. 2016) and one (*An. daciae*; Kronefeld et al. 2014b) delineated from a close relative (*An. messeae*) only some years ago (Nicolescu et al. 2004).

In the Czech Republic, no nationwide studies have been carried out recently, but data from several regional studies are available (e.g. Rettich et al. 2007; Votýpka et al. 2008; Šebesta et al. 2010, 2012a, b). Data from Liechtenstein are lacking, but as this small principality is completely surrounded by Austrian and Swiss territory, it seems reasonable to assume that no mosquito species other than in Austria and Switzerland are present.

Fig. 5.1 'Central Europe' according to the definition used in this contribution

In total, 65 culicid species belonging to the 7 genera *Aedes, Anopheles, Coquillettidia, Culex, Culiseta, Orthopodomyia* and *Uranotaenia* are represented in the defined Central European culicid fauna.

The taxonomy used in this contribution follows recent work by Wilkerson et al. (2015), as suggested to be currently applied by Reisen (2016). The phylogeny, systematics and use of taxonomic names within the family Culicidae have long been subjects of uncertainty and controversial discussion. Taxonomic revisions by Reinert et al. (2004, 2006, 2009), introducing numerous new genera for aedine mosquitoes (tribe Aedini) based on morphological characters, did not generally become accepted or were even rejected (Edman 2005).

5.4 Invasive Mosquito Species in Central Europe

Important species having recently become invasive in Central Europe belong to the genus *Aedes*, subgenera *Stegomyia* and *Hulecoeteomyia*: *Ae.* (*Stegomyia*) *albopictus, Ae.* (*Hulecoeteomyia*) *japonicus* and *Ae.* (*Hulecoeteomyia*) *koreicus*. These species originate from the Asian-Pacific region and are globally displaced by the international trade with used tyres, water-holding machinery and ornamental plants, such as 'lucky bamboo' (*Dracaena* sp.) (Reiter and Sprenger 1987; Madon et al.

2002; Derraik 2004; Hofhuis et al. 2009). Gravid females of these species are attracted to tyres collecting rain water when stored under the open sky and attach their eggs to the humid substrate above the waterline. The larvae hatch when the water level rises and the eggs are flooded, which may take place only after shipment to other regions of the world by additional rainwater. This had happened in France, Belgium and the Netherlands (Schaffner et al. 2001b, 2004; Scholte et al. 2012).

In the case of machinery and ornamental plants, larvae have often hatched and developed already at the time of importation (Scholte et al. 2007; Derraik 2004). Attempts to prevent their introduction by replacing the water to keep fresh the lucky bamboo by a gel substrate were not completely successful (Scholte et al. 2008; Demeulemeester et al. 2014).

Intracontinental transportation and displacement of invasive mosquito species may take place by vehicle transport of both eggs and adults. Thus, the movement of used tyres (eggs) as well as of horses in trailers (adults) is considered a major mode of passive *Ae. japonicus* spread in the USA (Scott et al. 1999), while adult mosquitoes following people into cars on their search for a blood meal are regularly introduced from southern to more northerly European countries (Kampen et al. 2013a).

The thermophilic Asian tiger mosquito *Ae. albopictus* was first found in Europe in the late 1970s in Albania (Adhami and Reiter 1998) but has obviously not spread from there. Only a decade later, this species was discovered reproducing in Genoa and Padua, Italy (Sabatini et al. 1990; Dalla Pozza and Majori 1992). The population from Padua is supposed to be the origin of the dispersal across southern Europe (Knudsen et al. 1996). *Aedes albopictus* has now been reported from 26 European countries, in at least 19 of which it is established (Medlock et al. 2015). Central European countries affected are Austria (Seidel et al. 2012), Belgium (Schaffner et al. 2004), the Czech Republic (Šebesta et al. 2012a; b), Germany (e.g. Kampen et al. 2013a; Werner and Kampen 2015), Hungary (Zöldi et al. 2016), the Netherlands (Scholte et al. 2012) and Switzerland (Flacio et al. 2015). Following Switzerland, Germany has recently reported repeated overwintering (Pluskota et al. 2016; Walther et al. 2017), thus being the first country north of the Alps where establishment has occurred. *Aedes albopictus* is considered an efficient vector of many viruses, including dengue and Zika viruses, and dirofilarial worms (Gratz 2004; Paupy et al. 2009; Heitmann et al. 2017).

In contrast to *Ae. albopictus*, *Ae. japonicus* is a species well adapted to temperate climates. Not surprisingly, it has succeeded in establishing in various parts of Europe (Kampen and Werner 2014). With a total of six separate populations, it presently occurs in Austria (Seidel et al. 2012, 2016a), Croatia (Klobučar et al. 2017), France (Krebs et al. 2014), Germany (Becker et al. 2011; Kampen et al. 2012; Werner and Kampen 2013; Zielke et al. 2016), Hungary (Seidel et al. 2016a), Italy (Seidel et al. 2016b) and the Netherlands (Ibáñez-Justicia et al. 2014). A seventh small population that had kept surviving in Belgium for several years (Versteirt et al. 2009) is supposed to be eradicated after implementing control measures (Versteirt et al. 2017).

Aedes japonicus has been found infected in the field with Japanese encephalitis, West Nile and La Crosse viruses, while it has been shown to be vector-competent for several viruses, including Japanese encephalitis, West Nile, dengue, chikungunya and Rift Valley fever viruses, in the laboratory (Kampen and Werner 2014; Harris et al. 2015).

Of the third Asian *Aedes* species established in Europe, *Ae. koreicus*, populations have been detected in Belgium (Versteirt et al. 2012), northern Italy (Capelli et al. 2011) and southern Switzerland (Suter et al. 2015). It is not yet clear whether a specimen collected in Germany in 2015 (Werner et al. 2016) had been introduced as that or must be ascribed to a hitherto undetected local population. *Aedes koreicus* is supposed to be a vector of various arboviruses (Cameron et al. 2010), has been found infected in the field with Japanese encephalitis virus and was shown to be vector-competent for dirofilarial worms in the laboratory (Werner et al. 2016).

Although after decades of absence *Ae. aegypti* has recently regained a foothold in Europe, i.e. on the Island of Madeira (Portugal), on the eastern Black Sea coast (southern Russia, Abkhazia) and in northeastern Turkey (Almeida et al. 2007; Akiner et al. 2016; Ganushkina et al. 2016), this species is not supposed to be able to establish in more northern parts of Europe in the near future due to its pronounced thermophily (Otero et al. 2006). Introduction, however, does occur (Scholte et al. 2010; Ibáñez-Justicia et al. 2016; Dallimore et al. 2017), and reproduction has also been observed, although indoors under subtropical conditions (Kampen et al. 2016).

There are other thermophilic mosquito species, though, newly emerging in some Central European countries, e.g. *An. hyrcanus* in Austria, the Czech Republic, Hungary and Slovakia (Tóth 2003; Halgoš and Benková 2004; Votýpka et al. 2008; Lebl et al. 2013), *Cs. longiareolata* in Austria and Germany (Seidel et al. 2013; Kampen et al. 2017) and *An. petragnani* in Germany (Becker et al. 2016; Kampen et al. 2017), possibly facilitated by climate warming. *Anopheles hyrcanus* is a potential vector of several pathogens including malaria parasites while *An. petragnani* is not regarded a vector. The transmission potential of *Cs. longiareolata* for viruses and *F. tularensis* is just speculative (van Pletzen and van der Linde 1981).

5.5 Potential Vectors Belonging to Species Complexes

Culicid species of the genus *Anopheles* are generally reduced to their roles in the transmission of malaria parasites. In fact, this genus does not only contain the vectors of human-pathogenic plasmodia, but there is evidence that they may transmit further pathogens, e.g. viruses. However, when searching the literature for pathogen identification in the mosquitoes and vector studies, one is confronted again and again with the problem of species complexes and designation of species. In some cases, the enigma of sibling species had just not been resolved at the time of reporting so that the authors did not know better while in other cases, the authors mark their data with question marks, indicating that they are uncertain about the sibling species in question. In many cases, however, the literature is just imprecise. Thus, it often remains unclear in the old literature whether the whole complex (*An. maculipennis* s.l., *An. claviger* s.l.) is meant or whether a species discrimination was performed and the eponymous species (*An. maculipennis* s.s., *An. claviger* s.s.) is referred to.

Additional problems arise when cryptic species have only recently been detected, such as *An. daciae* in the *An. maculipennis* complex, which was separated from its sibling *An. messeae* only in 2004 (Nicolescu et al. 2004). Sinka et al. (2010)

suggested that malaria cases formerly attributed to parasite transmission by *An. messeae* may indeed be due to *An. daciae*, given a documented relevance of *An. messeae* as a vector only in eastern Europe and the Balkans (Jetten and Takken 1994). The delineation of *An. daciae* from *An. messeae* has resulted in a situation that questions the validity of older literature describing the biological characteristics of *An. messeae*. Possibly, *An. daciae* or a mixture of *An. daciae* and *An. messeae* were hidden behind the old taxon name.

In all doubtful cases, the information given in the literature has been assigned to the complex as a whole for the purpose of this contribution.

In the *Cx. pipiens* complex, this contribution differentiates between the accepted species *Cx. pipiens* and *Cx. torrentium* but not between the *Cx. pipiens* biotypes *pipiens* and *molestus* although these are known to display significant biological differences (e.g, Fonseca et al. 2004).

5.6 General Considerations of 'Vector Potential'

Pathogen detection in field-collected specimens of a haematophagous arthropod as a clue for vector competence needs to be critically assessed. It certainly does not exclude vector competence but, in the first place, only suggests the imbibement of a pathogen and—depending on the detection procedure and technique (processing of certain body parts or the complete mosquito, microscopical or genetic detection of the pathogen)—does not necessarily mirror the ability of the pathogen to develop in the mosquito and use it as a biological vector. For being transmitted biologically, the ingested pathogen has to pass the midgut epithelium (i.e. to infect the mosquito), to disseminate, to replicate or maturate and to finally invade the body parts of the female mosquito from where release and transmission take place. Thus, the demonstration of virus or plasmodia in the salivary glands or of L3 metacyclic filarial larvae in the head or thorax of the mosquito is generally taken as evidence for vector competence. But even in these cases, transmission does not always occur (c.f., Jupp 1985). Virus particles in the salivary glands, for example, might just not be released into the salivary ducts due to a 'salivary gland escape barrier' (Hardy et al. 1983). Therefore, experimental demonstration of pathogen release during feeding, e.g. on a membrane, a cotton wool pad, into a buffer solution or to a vertebrate host, is the only valid proof of vector competence.

For the evaluation of the vector role of a mosquito species, it is of utmost importance to distinguish between vector competence and vector capacity. Vector competence is the principal ability of a vector to acquire and transmit a pathogen (Beerntsen et al. 2000). Thus, it is an innate, genetically fixed characteristic of a species which is specific for a pathogen species or even a particular strain of a pathogen species.

Basically, vector competence is the evolutionary result of mutual adaptation of the haematophagous insect and the pathogen, although arthropod species with physiological and biochemical features similar to the natural vector may also happen to be vector-competent, even if they, or their ancestors, had never had contact with the pathogen before. On the other hand, spontaneous mutations can modify the degree

of vector competence. It could be shown, for example, that a certain mutant strain of chikungunya virus, causative for the 2005–2006 epidemic on La Réunion Island, is transmitted much more efficiently than a nonmutant virus strain by *Ae. albopictus*, but not by *Ae. aegypti* (Tsetsarkin et al. 2007). Likewise, vector competence can vary from population to population of the same arthropod species under the same conditions (e.g. Weng et al. 1997). Principal vector competence given, efficiency of transmission can also be influenced by the temperature to which an infected arthropod (mosquito) is exposed during the extrinsic incubation period of the pathogen. Moreover, vector competence can be conferred or modified during larval development by stressors such as temperature, larval density, exposure to insecticides or food shortage (e.g. Turell 1993; Muturi and Alto 2011; Muturi et al. 2012).

As opposed to vector competence, vector capacity describes the de facto role of a vector-competent mosquito in a certain environment. According to Reisen (1989), vector capacity refers to 'potential new infections disseminated per case per day by each blood host presuming that all infected mosquito females become infective'. It takes into account vector and blood host density, blood host preference, biting rate, duration of extrinsic incubation period of the pathogen, probability of the vector to become infective and duration of its infective life (Reisen 1989). Thus, a vector-competent mosquito species may but does not necessarily need to be a vector.

5.7 Table

The selection of pathogens in Table 5.1 was made on the basis of these (1) to be of major public or animal health relevance (i.e. to have been linked to vertebrate infection and disease) and (2) to either have been shown to circulate in Europe or, if absent, to putatively use mosquito species occurring in Central Europe as vectors, according to field or laboratory findings. In all but one pathogen listed, symptomatic disease cases have been described after infection, either in humans or animal vertebrates. Lednice virus is the only pathogen mentioned not known to cause symptoms in vertebrates, including its avian hosts (Hubálek 2008). Dirofilarial infections compiled in Table 5.1 include both etiologic agents of canine dirofilariasis, *D. immitis* and *D. repens*.

Original literature is cited in Table 5.1 whenever possible but reduced to one reference article per pair of mosquito-pathogen species, although in some cases several reports exist. In very few cases, where original literature was not available or could not be identified, review documents are referred to as long as these seem to be credible (e.g. Sazonova (1965) for the linkage of *F. tularensis* and CDC (2018) for the linkage of West Nile virus with some mosquito species).

Some mosquito/pathogen combinations cited in general reviews, guides and catalogues without references, which could not be verified, e.g. *Ae. excrucians*/Batai virus, *Ae. sticticus*/Inkoo virus, *Ae. vexans*/Lednice virus, *An. hyrcanus*/Sindbis virus, *Cx. mimeticus*/West Nile virus, *Cs. longiareolata*/West Nile virus and *Cs. morsitans*/*Dirofilaria* sp. (e.g. Hubálek and Halouzka 1996; Schaffner et al. 2001a; ECDC 2014), were not compiled. Likewise, the sole linkage of the high

Table 5.1 Documented links between mosquito species occurring in Central Europe and pathogens

Species	BATV	CHIKV	DENV	EEEV	INKV	JEV	LACV	LEDV	RVFV	SSHV	SINV	SLEV	TAHV	USUV	WEEV	WNV	YFV	ZIKV	*Francisella tularensis*	*Plasmodium* spec.	*Dirofilaria* spec.	References
Aedes albopictus		F[1] T[2]	T[3] T[4] O[5]	F[6] T[7]		F[8] T[9] O[10]	F[11] T[12] O[13]		T[14]		T[15]	T[16] O[17]		F[18]	T[19]	F[20] T[21] O[22]	T[23]	F[24] T[25]			F[26] I[27]	[1]Bonilauri et al. (2008) [2]Mangiafico (1971) [3]Rudnick and Chan (1965) [4]Mitchell et al.(1987) [5]Rosen et al.(1983) [6]Niebylski et al. (1992) [7]Sardelis et al.(2002a) [8]Vythilingam et al. (1995) [9]Weng et al. (1997) [10]Rosen et al. (1978) [11]Westbyet al. (2015) [12]Grimstad et al. (1989) [13]Tesh and Gubler (1975) [14]Turell et al.(1988) [15]Dohm et al. (1995) [16]Savage et al. (1994) [17]Hardy et al. (1980) [18]Veronesi et al. (2012) [19]Wang et al. (2012) [20]Noden et al.(2015) [21]Turell et al. (2001) [22]Baqar et al. (1993) [23]Johnson et al. (2002) [24]Grard et al. (2014) [25]Heitmannet al.(2017) [26]Lai et al. (2001) [27]Cancrini et al. (2003)
Aedes annulipes																F[1]			F[2]		F[3]	[1]Thelaus et al. (2014) [2]Szentpáli-Gavallér et al. (2014) [3]Şuleşco et al. (2016b)
Aedes behningi																					F	Şuleşco et al. (2016b)
Aedes cantans													F[1]	F[2]		F[3]			F[4]		F[5]	[1]Málková et al. (1974) [2]Becker et al. (2014) [3]Labuda et al. (1974) [4]Thelaus et al. (2014) [5]Şuleşco et al. (2016b)
Aedes caspius		I[1]							I[2] T[3]				F[4] T[5]	F[6]		F[7] T[8]			F[9]		F[10] I[11]	[1]Vazeille et al. (2008) [2]Moutailler et al. (2008) [3]Turell et al. (1996) [4]Bardos and Danielová (1959) [5]Bulychev et al. (1978) [6]Mancini et al. (2017) [7]Bagheri et al. (2015) [8]Akhter et al. (1982) [9]Sazonova (1965) [10]Latrofa et al. (2012) [11]Ferreira et al. (2015)
Aedes cataphylla										F												Iversen et al. (1973)
Aedes cinereus				F[1]						F[2]	F[3] T[4]		F[5]			F[6]			F[7]		I[8]	[1]Armstrong and Andreadis (2010) [2]McLean et al. (1974) [3]Francy et al. (1989) [4]Turell et al. (1990) [5]Málková et al. (1974) [6]Andreadis et al. (2004) [7]Thelaus et al. (2014) [8]Arnott and Edman (1978)
Aedes communis	F[1]				F[2] O[2]					F[3] T[5] O[4]	O[2]	T[2]	F[6] T[6]						F[10]			[1]Francy et al. (1989) [2]Mitchell et al. (1993) [3]Tingström et al. (2016) [4]McLean et al. (1974) [5]McLean et al. (1977b) [6]Belloncik et al. (1982) [7]McLean et al. (1978) [8]Traavik et al. (1985) [9]Pchelkina and Seledtsov (1978) [10]Triebenbach et al. (2010)
Aedes cyprius																						
Aedes detritus		I[1]							I[2]				F[3]	F[4]		T[5]					I[6]	[1]Vazeille et al. (2008) [2]Moutailler et al. (2008) [3]Li et al. (2010) [4]Mancini et al. (2017) [5]Blagrove et al. (2016) [6]Ferreira et al. (2015)
Aedes diantaeus											O[1]		O[2]									[1]Tingström et al. (2016) [2]Traavik et al. (1978)
Aedes dorsalis						T[1]	F[2]					F[3] T[4]	F[5]		F[6] T[7]	F[8] T[9]					I[10]	[1]Reeves and Hammon (1946) [2]Sudia et al. (1971) [3]Emmons et al. (1985) [4]Chamberlain et al. (1959)

F pathogen demonstrated in/isolated from field-collected specimens; *I* demonstration of the pathogen in the body part of the mosquito from where transmission takes place (salivary glands for viruses and plasmodia, thorax or base of head for dirofilarial worms) or of mature third-stage dirofilaria in Malpighian tubules; *O* transovarial (vertical) transmission; *T* experimental transmission during feeding or salivation; *BATV* Batai virus; *CHIKV* chikungunya virus; *DENV* dengue virus (all serotypes); *EEEV* eastern equine encephalitis virus; *INKV* Inkoo virus; *JEV* Japanese encephalitis virus; *LACV* La Crosse encephalitis virus; *LEDV* Lednice virus; *RVFV* Rift Valley fever virus; *SSHV* snowshoe hare virus; *SINV* Sindbis virus; *SLEV* St. Louis encephalitis virus; *TAHV* Tahyna virus; *USUV* Usutu virus; *WEEV* western equine encephalitis virus; *WNV* West Nile virus; *ZIKV* Zika virus

Table 5.1 (continued)

																						[7]Cao et al. (2011) [8]Smart et al. (1972) [9]Kramer et al. (1998) [6]CDC (2018) [6]Goddard et al. (2002) [10]Ernst and Slocombe (1984)
Aedes esoensis						F																Sazonova (1965)
Aedes euedes																						
Aedes excrucians									T[1]	F[2]	T[3]		F[4]			F[5]			F[6]		I[7]	[7]Gargan et al. (1988) [2]Belloncik et al. (1982) [3]Turell et al. (1990) [4]Mitchell et al. (1993) [5]Vinograd et al. (1982) [6]Sazonova (1965) [7]Arnott and Edman (1978)
Aedes flavescens													F[1] T[2]			F[3]					F[4]	[1]Aspöck and Kunz (1967) [2]Pchelkina and Seledtsov (1978) [3]Anderson et al. (2015) [4]Şuleşco et al. (2016b)
Aedes geminus																						
Aedes geniculatus																T[1]	T[2]				F[3] T[4] I[5]	[1]Vermeil et al. (1960) [2]Roubaud et al. (1937) [3]Şuleşco et al. (2016b) [4]Roubaud and Colas-Belcour (1937) [10]Silaghi et al. (2017)
Aedes hexodontus					F[1]					F[2]			F[3]									[1]Mitchell et al. (1993) [2]McLean et al. (1977a) [3]Traavik et al. (1978)
Aedes hungaricus																						
Aedes intrudens										F[1]									F[2]			[1]Ritter and Feltz (1974) [2]Thelaus et al. (2014)
Aedes japonicus		T[1]	T[1]	T[2]		T[3] O[2]	F[4] T[5]		T[6]			T[7]				F[8] T[9]					I[10]	[7]Schaffner et al. (2011) [5]Sardelis et al. (2002a) [3]Takashima and Rosen (1989) [1]Westby et al. (2015) [5]Sardelis et al. (2002b) [6]Turell et al. (2013) [5]Sardelis et al. (2003) [6]CDC (2018) [6]Sardelis and Turell (2001) [10]Silaghi et al. (2017)
Aedes koreicus						F[1]															I[2]	[1]Miles (1964)

																						[9]Montarsi et al. (2015)
Aedes leucomelas																			F			Thelaus et al. (2014)
Aedes nigrinus																						
Aedes nigripes																						
Aedes pionips																						
Aedes pulcritarsis																						
Aedes pullatus																						
Aedes punctor	F[1]				F[2]					F[3]	O[4]		F[5] T[4]			T[7]			F[8]		T[9]	[7]Vinograd et al. (1973) [4]Mitchell et al. (1993) [5]McLean et al. (1977a) [4]Tingström et al. (2016) [6]Traavik et al. (1985) [6]Pchelkina and Seledtsov (1978) [7]Vermeil et al. (1960) [9]Lundström et al. (2011) [2]Roubaud and Colas-Belcour (1937)
Aedes refiki																						
Aedes riparius																					F	Şuleşco et al. (2016b)
Aedes rossicus														F[1]		F[2]						[1]Nowotny and Weissenböck (2004) [2]Hubálek et al. (2010)
Aedes rusticus																						
Aedes sticticus				F[1]									F[2] T[3]			F[4]			F[5]		F[6] I[7]	[1]Anderson et al. (1961) [1]Traavik et al. (1978) [3]Danielová (1966) [4]Andreadis et al. (2004) [5]Lundström et al. (2011) [5]Şuleşco et al. (2016b) [7]Ernst and Slocombe (1984)
Aedes surcoufi																						
Aedes vexans	F[1]	F[2]		T[3] T[4]		T[5]	F[6]		F[7] T[8] O[9]	F[10]		F[11] T[12]	F[13] I[14] T[15] O[16]	F[17]	F[18] T[19]	F[20] T[21]		T[22]	F[23]		F[24] I[25]	[7]Gould et al. (1968) [2]Guo et al. (2015) [2]Armstrong and Andreadis (2010) [8]Davis (1940) [3]Hodes (1946) [8]Sudia et al. (1971) [2]Fontenille et al. (1995) [1]Turell et al. (2008) [2]El Hadi Mohamed et al. (2013) [17]Anderson et al. (2015) [11]Emmons et al. (1974) [10]Hammon and Reeves (1943)

(continued)

Table 5.1 (continued)

																						[15]Bardos and Danielová (1959) [16]Danielová (1968) [16]Šimková et al. (1960) [16]Danielová and Ryba (1979) [17]Nowotny and Weissenböck (2004) [18]Burroughs and Burroughs (1954) [19]Kelser (1935) [20]Feiszli et al. (2010) [21]Goddard et al. (2002) [22]O'Donnell et al. (2017) [23]Lundström et al. (2011) [24]Latrofa et al. (2012) [25]Ernst and Slocombe (1984)
Anopheles algeriensis																				I		Sergent and Sergent (1905)
Anopheles claviger s.l.																			F^1	T^2	F^3 T^4	[1]Sazonova (1965) [3]Hargreaves (1923) [4]Şuleşco et al. (2016a) [4]Grassi and Noé (1900)
An. claviger	F^1												T^2	F^3					F^4	F^5		[1]Traavik et al. (1985) [2]Pchelkina and Seledtsov (1978) [3]Becker et al. (2014) [4]Thelaus et al. (2014) [5]Gutsevich et al. (1974)
An. petragnani																					F	Mantovani and Restani (1965)
Anopheles hyrcanus						F^1							F^2		F^3	F^4			F^5	I^6	F^7 I^8	[1]Miles (1964) [4]Lvov et al. (1972) [4]Lu et al. (2001) [4]Lvov et al. (2004) [5]Sazonova (1965) [6]Jackson (1951) [7]Djadid et al. (2009) [8]Feng (1930)
Anopheles maculipennis s.l.	F^1									F^2		F^3	F^4			F^5			F^6	F^7 T^8	F^9 I^{10}	[1]Aspöck and Kunz (1968) [2]Jöst et al. (2010) [3]Aspöck et al. (1970) [4]Jöst et al. (2011) [5]Filipe (1972) [6]Thelaus et al. (2014) [7]Barber and Rice (1935) [8]Roubaud (1918) [9]Czajka et al. (2014) [10]Ferreira et al. (2015)
An. atroparvus																				F^1	I^2	[1]Swellengrebel et al. (1938)

																				T^2		[2]Weyer and Bock (1939) [2]Kuzmin et al. (2005)
An. daciae																				?	F^1	[1]Sinka et al. (2010) [1]Kronefeld et al. (2014a)
An. maculipennis																				F^1 I^2	I^3	[1]Lorincz (1937) [2]Barber and Rice (1935) [3]Azari-Hamidian et al. (2009)
An. messeae	F^1															F^1				F^2 I^3		[1]Lvov et al. (2004) [2]Swellengrebel et al. (1938) [3]Zhukov and Krasikova (1942)
Anopheles plumbeus																T^1				I^2	F^3	[1]Vermeil et al. (1960) [2]Eling et al. (2003) [3]Roubaud and Colas-Belcour (1937)
Coquillettidia richiardii	F^1												F^2			F^3			F^4		F^5 I^6	[1]Aspöck and Kunz (1968) [2]Aspöck and Kunz (1967) [3]Szentpáli-Gavallér et al. (2014) [4]Thelaus et al. (2014) [5]Şuleşco et al. (2016b) [6]Cancrini et al. (2006)
Culex hortensis														F								Nowotny and Weissenböck (2004)
Culex martinii																						
Culex mimeticus																						
Culex modestus									F^1		F^2		F^3	F^4		F^5 T^6			F^7		F^8 I^9	[1]Málková et al. (1974) [2]Alkhovsky et al. (2014) [3]Cao et al. (2011) [4]Becker et al. (2014) [4]Lvov et al. (2004) [5]Balenghien et al. (2008) [6]Sazonova (1965) [6]Şuleşco et al. (2016b) [6]Rossi et al. (1999)
Culex pipiens	F^1					F^2 T^3			T^4		F^5 T^6	F^7 T^8	F^9 T^{10}	F^{11}	F^{12} T^{13}	F^{14} T^{15} O^{16}			F^{17}		F^{18} I^{19}	[1]Becker et al. (2014) [3]Kim et al. (2015) [4]Reeves and Hammon (1946) [4]Turell et al. (1996) [5]Jöst et al. (2010) [6]Dohm et al. (1995) [7]Tsai et al. (1988) [8]Reeves et al. (1942) [9]Arcan et al. (1974) [10]Pchelkina and Seledtsov (1978) [11]Jöst et al. (2011)

Table 5.1 (continued)

																						[12]Emmons et al. (1979) [13]Wang et al. (2007) [14]Szentpáli-Gavallér et al. (2014) [15]Balenghien et al. (2008) [16]Nelms et al. (2013) [17]Sazonova (1965) [18]Latrofa et al. (2012) [19]Rossi et al. (1999)
Culex territans				F[1]					T[2]					F[3]		F[4]						[1]Morris et al. (1973) [2]Gargan et al. (1988) [3]Nowotny and Weissenböck (2004) [4]CDC (2018)
Culex theileri									F[1] T[1]		F[2] T[2]					F[2] T[3]					F[1] I[1]	[1]McIntosh et al. (1980) [2]McIntosh et al. (1976) [3]Jupp et al. (1972) [4]Santa-Ana et al. (2006) [5]Ferreira et al. (2015)
Culex torrentium											F[1] T[2]					F[3]						[1]Jöst et al. (2010) [2]Lundström et al. (1990) [3]Leggewie et al. (2016)
Culiseta alaskaensis																			F			Triebenbach et al. (2010)
Culiseta annulata	F[1]												F[2] T[2] O[2]	F[2]							F[3]	[1]Becker et al. (2014) [2]Danielová et al. (1972) [3]Danielová (1972) [4]Bárdoš et al. (1978) [5]Nowotny and Weissenböck (2004) [6]Czajka et al. (2014)
Culiseta fumipennis																						
Culiseta glaphyroptera																						
Culiseta longiareolata																					F	Şuleşco et al. (2016b)
Culiseta morsitans				F[1]							F[2]					F[3]						[1]Morris et al. (1973) [2]Francy et al. (1989) [3]CDC (2018)
Culiseta ochroptera																						
Culiseta subochrea																						
Orthopodomyia pulcripalpis																						
Uranotaenia unguiculata																F[1]					F[2]	[1]Kemenesi et al. (2014) [2]Şuleşco et al. (2016b)

abundance of a certain mosquito species in an area or during a period of mosquito-borne disease occurrence in the absence of other species, or the majority, but not exclusiveness, of a certain mosquito species in a pool tested positive for a pathogen was not taken as evidence for that species to be infected or the vector. Also, documentations of mixtures of two mosquito species without a preference for one or the other as the assumptive virus carrier were ignored. Thus, *Ae. dorsalis, Cq. richiardii* and *Ae. communis* are not linked to Sindbis virus (Berezin et al. 1971; Lvov et al. 1984), and *Ae. annulipes* is not linked to Tahyna virus in the table (Aspöck and Kunz 1967).

For similar reasons, it was decided not to list *Ae. cantans, Ae. excrucians* and *Ae. flavescens* as demonstrated vectors of Tahyna virus (Danielová 1966) and *Ae. communis* as a demonstrated vector of Sindbis virus (Turell et al. 1990), although all species showed virus multiplication after a period of time long enough after the infectious blood meal to become infective (>14 days and 8–14 days, respectively). However, mosquitoes were assayed completely, so that evidence of virus dissemination into the salivary glands was missing.

In the table, it is differentiated between (1) the simple demonstration of a pathogen in the (usually field-collected) mosquito species, (2) the demonstration of a pathogen in the organ/body part of the mosquito from where transmission takes place (salivary glands for viruses and plasmodia, head or basis of thorax for dirofilarial worms) or of maturity of the pathogen in the mosquito organ where it develops (Malpighian tubules for dirofilarial worms), (3) the demonstration of pathogen transmission during feeding and (4) the demonstration of transovarial (vertical) transmission of a pathogen to the next generation after infection of the female parent.

Literature evidence for option (2) is not listed if option (3) is documented. Viral salivary gland infection after mere experimental parenteral inoculation is not considered as the mesenteron, which is an efficient barrier to many viruses (Hardy et al. 1983), is circumvented by this mode of infection. Likewise, transovarial transmission is ignored after intrathoracical inoculation, unless further studies exist where both natural infection and transmission by feeding were demonstrated, indicating ability of the virus to pass the mesenteron (e.g. Tesh and Gubler 1975; Rosen et al. 1978; Baqar et al. 1993).

5.8 Conclusions

Although—despite most extensive literature research—the table might probably not be complete, it becomes obvious that pathogen transmission by the listed mosquito species has most often been shown in the invasive species *Ae. albopictus* and *Ae. japonicus*, followed by the native common species *Cx. pipiens, Cx. theileri, Ae. communis, Ae. dorsalis, Ae. geniculatus, Ae. punctor* and *Ae. vexans*. The latter species, however, also have a broad distribution outside Central Europe, e.g. in Russia (all species) and North America (not *Ae. geniculatus* and *Cx. theileri*) or South Africa (*Cx. theileri*), and may therefore have generally been more interesting objects of transmission studies than species restricted to Central Europe.

The finding of pathogens in field-collected specimens has been documented in numerous additional mosquito species, but the vector competence of these can only be elucidated within the scope of experimental studies. These might be hampered by the fact that many mosquito species cannot be reared in the laboratory, but as a compromise, larvae can be collected in the field for performing infection and transmission experiments with the emerging adults as long as these are willing to feed under artificial conditions.

Also, the possibility and frequency of transovarial transmission and its impact on maintaining a natural enzootic cycle of a mosquito-borne pathogen devoid of vertebrate hosts need further examination both in the laboratory and in the field.

If possible, vector competence studies should include different mosquito populations of the same species and different pathogen strains and be done under different biotic and abiotic conditions, thus taking into account differences in vector competence/potential and climatic changes. Only with the help of these data, actual vector roles of the various Central European mosquito species can be assessed.

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Chapter 6
Essential Oils from Aromatic and Medicinal Plants as Effective Weapons Against Mosquito Vectors of Public Health Importance

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Abstract The fight against mosquito-borne diseases has recently seen the failure of control programmes based on synthetic chemical treatments to combat larvae and adults of mosquito vectors. This has led to several problems linked to residual substances causing a detrimental impact on environment and human health and to the development of resistance in mosquitoes. In this scenario, new eco-friendly and alternative strategies for the management of mosquito-borne diseases come from the use of plant essential oils (EOs). These are complex mixtures of small, volatile and lipophilic compounds, mostly belonging to monoterpenoids, sesquiterpenoids and phenylpropanoids, produced by aromatic plants belonging to several botanical families such as Apiaceae, Asteraceae, Geraniaceae, Lamiaceae, Lauraceae, Myrtaceae, Poaceae, Rutaceae, Verbenaceae and Zingiberaceae. An important ecological role played by EOs is defending plants from several enemies such as bacterial and fungal pathogens, viruses, insects and parasites. EOs represent ideal candidate ingredients to be incorporated in insecticidal formulations since scientific evidences have documented their efficacy against larvae and adults of several mosquitoes (e.g. *Anopheles*, *Aedes* and *Culex*) even at low doses (<50 ppm), the multiple mode of action and wide spectrum of efficacy, the low toxicity on nontarget organisms and environment and the unlikely capacity to induce insect resistance. In this chapter, we gave an overview of the most important EOs obtained from commercially important botanical families with documented efficacy against mosquito vectors. Particular attention has been paid to highlight their strengths and weakness and the future challenges leading to the replacement of conventional insecticides by agrochemical companies.

Keywords Medicinal and aromatic plants · Essential oils (EOs) · Chemical composition · Mosquitoes · Mechanism of actions · Larvicidal activity · EO-based formulations

6.1 Introduction

The deleterious impact on environment and human health afforded by synthetic insecticides used until the second half of the twentieth century forced the regulatory authorities to reduce or delete the use of harmful substances from the arsenal of agrochemical companies. Main examples are given by DDT and methyl parathion, the former used to combat malaria vectors and the latter as a potent pesticide in crop protection, both completely abandoned in the 1970s as they were classified as carcinogenic and extremely toxic substances, respectively (Morgan 2004). These synthetic substances were replaced by others with minor impacts on human and animal health and environment. At the same time, the public opinion in wealthy countries looked at the natural alternatives from plant sources, the so-called botanical insecticides, with increasing interest. Therefore, since the 1960s, when the insecticidal azadirachtin, a limonoid isolated from the neem tree (*Azadirachta indica* A. Juss., Meliaceae), was discovered, natural products received particular attention by academic researchers looking for suitable alternatives to conventional insecticides.

Among natural products, plant essential oils (EOs) are interesting substances with an important perspective of use in the near future as effective botanical insecticides. EOs enjoy a long history of consumption as flavourings and fragrances, spices and medicines. Also, old uses to protect stored foodstuffs and to repel various insects have been documented. It is only relatively recently, however, that they become reliable tools in pest management science so that their commercialization has begun (Isman and Machial 2006). After the introduction of EO-based insecticides on the market at the beginning of the twenty-first century, the scientific research on EOs has seen an unpredictable progression. As an example, the percentage of all papers on botanical insecticides dealing with the effects of EOs increased from 8% in 2000 to 23% in 2012 (Isman and Grieneisen 2014). Overall, the worldwide leadership in scientific research on botanical insecticides, including EOs, belongs to a few countries such as India, China, Brazil and the USA (Isman and Grieneisen 2014). Regarding the patented EO-based formulations used as mosquito repellents, they have been manufactured mostly in China, Japan and Korea (Pohlit et al. 2011).

According to the *European Pharmacopoeia*, an EO is an '*Odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating*' (European Pharmacopoeia 2005). From this definition, it is clear that neither solvent extractions nor procedures adopted on an industrial scale using supercritical fluids allow to obtain a product classifiable as EO.

EOs are complex mixtures of volatile compounds produced by the plant secondary metabolism and consisting in relatively low-boiling point, low-molecular weight and lipophilic molecules. Notably, the EO composition is made up of hundreds of chemical constituents, and generally a few components (usually 3–5) occur in significant amounts so that they determine the EO quality by influencing the chemico-physical and biological properties of the EO itself. Nevertheless, minor compounds of EOs may play an important role and affect the overall bioavailability and bioactivity. EO composition is generally dominated by terpenoids such as monoterpenes and sesquiterpenes. As a matter of fact, these groups of secondary metabolites are the most cited in literature for their efficacy on insects (Boulogne et al. 2012). In addition, other classes of components occurring in EOs are represented by phenylpropanoids and aliphatic compounds. Therefore, each EO possesses a specific peculiarity (e.g. organoleptic, chemico-physical properties, mode of action, etc.) given by its own chemical profile. The chemical composition of EOs can be subjected to fluctuations due to different factors such as harvesting period, geographic origin, climate, genetics, chemotype, processing and extraction procedures (Holopainen and Gershenzon 2010).

To the best of our knowledge, about 3000 higher plants (also known as 'aromatic plants') are recognized as sources of EOs, among which 300 are very important on a commercial scale and used by the food, agronomic, pharmaceutic, cosmetic and perfume industries (CBI 2009a; Bakkali et al. 2008). Their main industrial applications are as flavouring of foodstuffs and beverages including liqueurs, as fragrances in perfumes and cosmetics and as antimicrobial agents in pharmaceutics and foodstuffs (Lubbe and Verpoorte 2011). It has been estimated that more than hundred thousand tonnes of EOs are produced per year, of which 90% is only for flavour and

fragrances industries (CBI 2009b). The major consumers are the USA, France, Germany, the UK and Japan (Holmes 2005).

Important factors favouring the spread of EO-based insecticides are (1) the worldwide availability of raw materials from which EOs are obtained at relatively low cost, (2) the high yields of EOs obtainable from cheap and available plant sources, (3) the relative ease of preparation and chemical characterization by gas chromatography coupled with mass spectrometry (Rubiolo et al. 2010), and iv) their acknowledged safety to humans (Isman et al. 2011). Currently, the most important hurdle limiting their diffusion and commercialization is the approval by more restricting regulatory organisms settled in the various countries.

6.2 Chemical Profile of Essential Oils (EOs)

EOs can be divided into three main groups on the basis of the chemical nature of their major chemical constituents. Definitely, the most frequent group is that of terpenoids, notably monoterpenes and sesquiterpenes, whereas diterpenes are less frequent. These compounds can be formed in the plant cell through two main biosynthetic pathways called mevalonate and methyl erythritol phosphate, respectively (Fig. 6.1) (Dewick 2002).

Both pathways give raise to C5 building blocks, namely, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) which, through head-to-tail condensations, originate the C10 (geranyl pyrophosphate, GPP) and C15 (farnesyl pyrophosphate, FPP) precursors of the monoterpene and sesquiterpene classes, respectively. The two pathways take place in different compartments of the plant cell, with the former occurring in the chloroplast and the latter in the cytoplasm

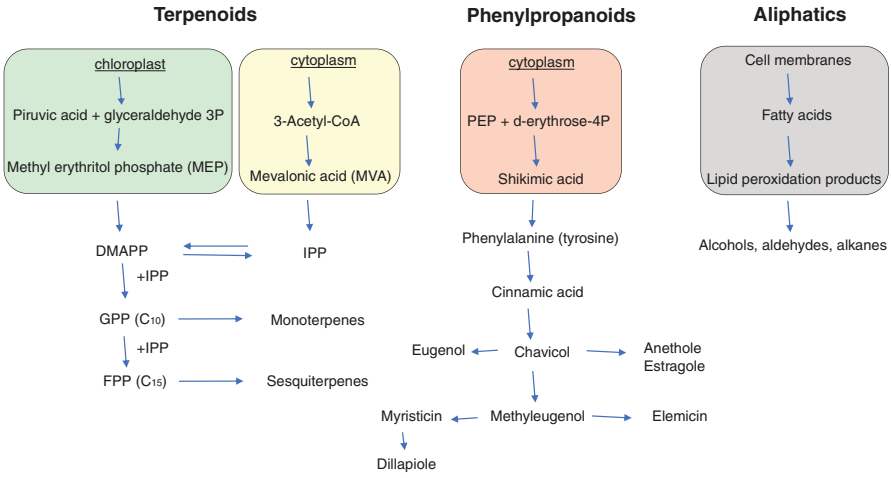


Fig. 6.1 Metabolic pathways in the plant cell leading to different groups of volatile compounds

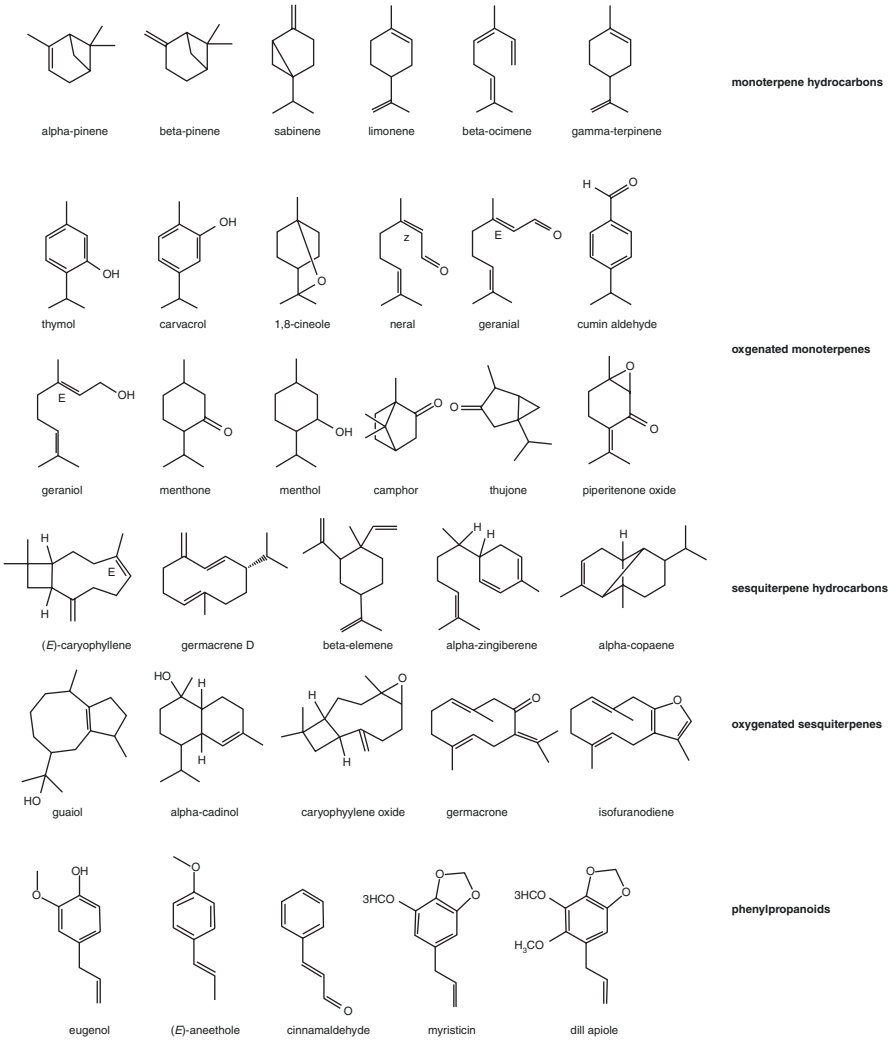


Fig. 6.2 Chemical structures of various EO constituents with known insecticidal activities

(Fig. 6.1). Specific enzymes acting on GPP and FPP give raise to linear, cyclic, bicyclic and tricyclic chemical structures (Fig. 6.2).

The simplest compounds are those with chemical formulas $C_{10}H_{16}$ (monoterpenes) and $C_{15}H_{24}$ (sesquiterpenes) given by hydrocarbons like α- and β-pinene, limonene, γ-terpinene, terpinolene, (*E*)-caryophyllene and germacrene D. However, their structures are rich of saturations which make them highly reactive and oxidable. Indeed, further oxidative, oxidoreduction and desaturation reactions can lead to the formation of alcoholic (-OH), aldehydic (-CHO), ketonic (=O), ester (-O-CO-) and phenolic ($-C_6H_5$-OH) functions. Some examples of these structures are given by geraniol, geranial, carvone, linalyl acetate and thymol, respectively (Fig. 6.2). Terpene-rich EOs

occur in the most evolved family of Angiosperms such as Lamiaceae (e.g. mint, sage, rosemary, thyme, oregano) and Asteraceae (e.g. absinth, huacatay, qinghao). Generally, monoterpenes have boiling points in the range 140–180°C, whereas sesquiterpenes are above 200°C. This makes all of them relatively volatile and non-persistent in the environment. The volatility renders them as ideal candidate as repellent agents but at the same time limits their application (Isman and Machial 2006).

On the other hand, some insecticidal EOs are dominated by phenylpropanoids such as eugenol (clove), cinnamaldehyde (cinnamon), anethole (fennel, star anise, anise) and myristicin (nutmeg, parsley, celery, carrot) (Fig. 6.2). These compounds are all formed via the shikimate pathway resulting in phenylalanine from which cinnamates and phenylpropenes can be formed (Fig. 6.1).

Lastly, EOs sporadically can be dominated by, e.g. chlorophyll and carotenoid degradation products, aliphatic alcohols, aldehydes and ketones which originate from lipid peroxidation. For instance, phytol is a linear diterpene alcohol constituting the tail of chlorophyll from which it is produced as degradation product (Maggi et al. 2009). Generally, EOs with high levels of phytol are poor in monoterpenes and sesquiterpenes. Another example is given by the secondary alcohol 1-octen-3-ol, the major volatile component of bastard balm (*Melittis melissophyllum* L.), which is formed via aerobic oxidation of linoleic acid by lipoxygenase followed by enzymatic cleavage (Maggi et al. 2010a). This compound is the key odorant of many species of edible fungi (Maggi et al. 2010b, 2012a). 1-Octen-3-ol is also a component of the human odour attracting the malaria mosquito *Anopheles gambiae*, thus potentially playing a role in the transmission of disease (Carey et al. 2010). Other artefact products that may occur in EOs are alkanes, especially linear C_{23}–C_{27} hydrocarbons, which derive from the degradation of waxy coatings on leaves and flowers of EO-poor species (Afshar et al. 2015). *n*-Alkane distribution may follow A and B patterns: the former includes even and odd alkanes in equal amounts (C_{22}–C_{28}) which originate from parenchyma tissues; the latter comprises mostly odd alkanes (C_{25}–C_{33}) which originate from epidermal tissues (Alves-Pereira and Fernandes-Ferreira 1998; Carriere et al. 1990). Generally, this kind of EOs is poorly interesting for the flavour and fragrance industry and biologically inert.

6.3 Mosquito-Borne Diseases

Among more than 3500 species of mosquitoes, about 100 are recognized as vectors of pathogens of human beings and other vertebrates (Pavela 2015). These species belong to three main genera of Culicidae (Diptera), namely, *Anopheles* Meigen, *Aedes* Meigen and *Culex* L. (Fig. 6.3) (Pohlit et al. 2011). Bites of these mosquitoes transmit important diseases such as malaria, dengue, yellow fever, West Nile, filariasis and Zika virus (Attar 2016; Benelli and Mehlhorn 2016; Benelli et al. 2016, 2017a; Nicoletti et al. 2016). These diseases cause high morbidity and death, mainly in the developing countries where they represent a major economic burden. However, in the last decades, several outbreaks of these diseases like chikungunya

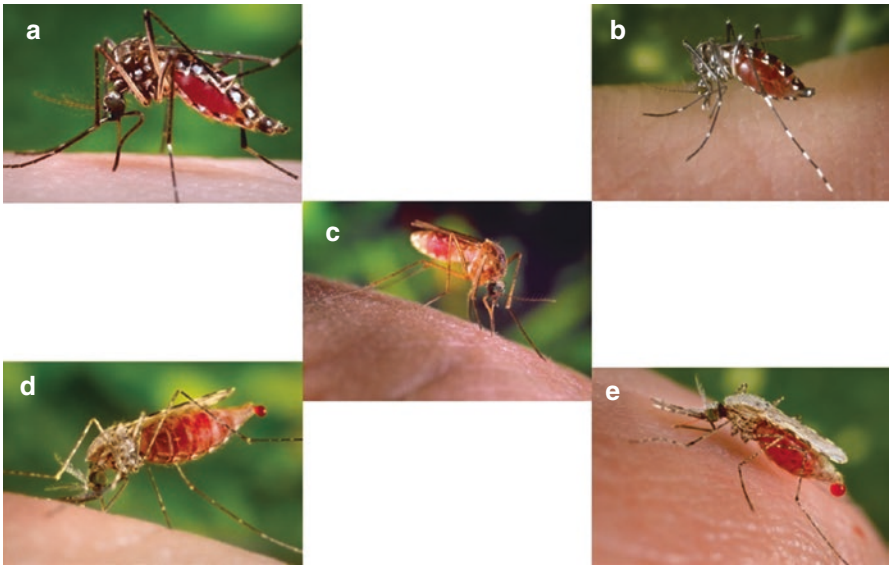


Fig. 6.3 Adults of mosquito vectors: (**a**) *Aedes aegypti*; (**b**) *Aedes albopictus*; (**c**) *Culex quinquefasciatus*; (**d**) *Anopheles gambiae*; (**e**) *Anopheles stephensi*

occurred in Europe (Rezza et al. 2007). Therefore, there is a need to find countermeasures to embank these new infectious threats in the next years.

Malaria is one of the most important mosquito-borne diseases causing approximately 250 million cases and more than one million deaths, affecting mainly children in Africa (WHO 2010). In the latter, some symptoms like severe myalgia, headache and fever are associated with malaria although they occur in cases of influenza (Waitumbi et al. 2010). Malaria is an infection caused by several protozoan species such as *Plasmodium falciparum* Welch, *P. vivax* Grassi & Feletti, *P. malariae* Feletti & Grassi and *P. ovale* Stephens (Pohlit et al. 2011). These parasites are transmitted to humans through the bites of infected *Anopheles gambiae* Giles, *An. arabiensis* Patton and *An. stephensi* Liston, among others (Wachira et al. 2014). Owing to the development of resistance in parasites and mosquito vectors towards conventional drugs and insecticides, malaria incidence has increased in the last decades (Kweka et al. 2016).

Females of *Aedes aegypti* L. and *Ae. albopictus* Skuse transmit dengue virus which caused severe epidemics throughout Africa, Asia and North America in the eighteenth century (Rigau-Pérez 2006). In addition, *Ae. aegypti* vectors other diseases such as yellow fever, chikungunya and Zika virus (Moreira et al. 2009). This mosquito is capable of adapting to different habitats linked with human settlements and lays eggs in wet places (Suresh et al. 2015). In tropical and subtropical regions, dengue strikes up to 400 million people every year. Its spread in some countries such as India, Pakistan and Bangladesh is associated with urbanization and population growth (Monath 2007). The main symptoms of this disease are fever, headache, myalgia, arthralgia, rash, nausea, vomiting and bleeding (Jelinek et al. 1997).

Another important tropical disease is lymphatic filariasis, also known as elephantiasis, which is mostly caused by the nematode *Wuchereria bancrofti* (Cobbold) in

turn transmitted by the bites of *Culex* species (Benelli 2015a; Benelli and Mehlhorn 2016). Among them, *Cx. quinquefasciatus* Say is definitely the most important vector in urban and rural areas of Asia (Chadee et al. 2002). In Africa, this vector can transmit the Rift Valley fever virus (Vadivalagan et al. 2017). It has been estimated that more than one million people in tropical regions are at risk to be infected by lymphatic filariasis (WHO 2014). The most common symptoms of these illnesses are pain and swelling to genitals, whereas lymphangitis, lymphadenitis and elephantiasis are less frequent (Wijers and McMahon 1976). To eradicate this plague, the WHO launched the 'Global Programme to Eliminate Lymphatic Filariasis' targeting its complete eradication by 2020 (WHO 2014). Besides humans, *Culex* species transmit pathogens and parasites including dog heartworm, West Nile virus and Eastern equine encephalitis to animals such as horses and dogs (Mehlhorn 2015).

Mosquito control is currently dealing with important challenges in integrated pest management programmes owing to the alarming development of insecticide resistance (Naqqash et al. 2016) and the little success of biocontrol strategies (Benelli and Mehlhorn 2016). In this scenario, the use of plant EOs may represent an alternative, intriguing and eco-friendly tool to control mosquito vectors (Govindarajan and Benelli 2016a, b; Pavela and Benelli 2016). EO constituents can be regarded as relatively selective and degradable in the environment and with minor or no side effects on beneficial organisms.

Actually, there are three main strategies to combat mosquito vectors. The first one and most widespread is to kill adults through the use of insecticides, insecticidal nets and insecticide-treated nets. The second one is to inhibit the bite of blood-sucking insects using repellents. However, this method often has a lower efficacy compared with the other ones and needs repeated applications of the repellent at regular intervals of time. The third one is the reduction of the population density through the application of insecticides into puddles, stagnant waters and aquatic habitats where larvae usually breed and complete their metamorphosis (Pavela 2015). In particular, these strategies are employed to reduce the transmission of malaria in urban and rural areas by decreasing the number of mosquitoes achieving adulthood. Owing to their low mobility, young instar larvae are attractive targets to control mosquito populations although this method is not desirable to be applied in rural areas (Pavela 2015).

Overall, EOs, wherever properly formulated, are ideal candidates to be employed as larvicides, ovicides, adulticidal and repellent agents. They can be used in integrated pest management programmes associated with conventional products in order to reduce the dose causing toxicity on nontarget organisms and environment and to avoid the development of insect resistance.

6.4 Insecticidal Activity of EOs

Since the beginning of their evolution, higher plants have evolved a plethora of defence mechanisms in order to defend themselves from a great number of enemies such as insects, herbivores and microbial pathogens. As a consequence, plants specialized in the synthesis of a variety of chemical substances, also known as

allelochemicals, acting towards these organisms and inhibiting their metabolism and behaviour (Rattan 2010). Vice versa, insects evolved an own strategy to overcome plant defences, and the final result was a coevolution between plants and insects which is still in progress. As a result, some chemical compounds produced by plants are toxic or repellent for some species of insects and attractive and beneficial for other ones (e.g. pollinators). Among the large array of allelochemicals (nowadays also known as secondary metabolites) produced by plants to interact with their surroundings, EOs are one of the most important in the evolution scale, being abundant in species belonging to the most evolved taxonomic groups of angiosperm dicotyledons (e.g. advanced Rosidae and Asteridae) (Gardner 1977).

EOs can be considered as important elements of the so-called constitutive chemical defence, in the sense that they are always present inside the plant (Wittstock and Greshenzon 2002). Less often, they are produced as a consequence of an herbivore attack (Karban and Myers 1989). In the first case, they are compartmentalized in specialized secretory tissues of epidermal or parenchymatic origin (e.g. glandular trichomes, translucent glands, ducts, vittae, lysogenic and schizogenic pockets) (Sangwan et al. 2001).

The biological effects displayed on mosquitoes by EOs depend on their mechanism of action although some of them are not completely elucidated. Generally, these effects can be classified into two main groups, namely, behavioural (e.g. repellent, antifeedant, inhibition of oviposition) and physiological (e.g. acute toxicity, inhibition of development and growth) (Isman 2017).

Overall, the insecticidal activity of EOs is strongly influenced by the lipophilicity and small size of their constituents that allow them to cross easily the insect surface, diffuse through the body and enter the cells. In addition, their high lipophilicity allows them to interact with behavioural, metabolic and physiological processes of insect (Jacobson 1989).

Generally, EOs cause neurotoxic effects in insects by interacting with acetylcholinesterase (AChE), gamma-aminobutyric acid (GABA) and octopaminergic receptors (Pavela and Benelli 2016). They are also able to neutralize the defensive system of insect by inhibiting the detoxification enzymes such as cytochrome P450-dependent monooxygenases (Wittstock and Greshenzon 2002). Last but not least, EOs are known to disrupt the cytoplasmic membrane causing leakage of intracellular components and structural alterations leading to cell death (Burt 2004).

Often the diverse mechanisms of action occur together since EOs show in their composition more than one active component exerting a specific mode of action on mosquito. Thus, synergistic effects resulting from the presence of several constituents with different chemical structures and different modes of action are frequent so that the crude EO exerts generally higher toxicity than that of its major components (Hummelbrunner and Isman 2001). Another advantage from the use of EOs is the absence of possible episodes of resistance in mosquitoes. This can be explained by their multiple mechanism of action, and this aspect reinforces their incorporation in insecticidal formulations, also in association with synthetic active compounds (Pavela and Benelli 2016). It is worth to note that frequently also the minor components may be involved and contribute to the whole insecticidal effects of EOs. In fact, whether such a component is slightly or not at all effective on a specific insect when administered alone, it increases its efficacy when mixed with other components (Pavela 2015).

Therefore, it is very important to characterize in detail the EO chemical composition by an accurate gas chromatographic analysis. In our opinion, a very important concept to keep in mind in this field should be the preparation of EO mixtures having the same chemical profile in terms of quantitative ratios of their chemical constituents, rather than focusing on the absolute concentrations of their major components. EOs are not a simple sum of several chemical compounds but a different entity given by complex and little understood interactions between their major and minor constituents.

6.4.1 Larvicidal Activity

Currently, one of the most used strategies to limit adult populations of mosquitoes in urban areas is to target the larval stages in their breeding sites with larvicides (WHO 2012). In this respect, EOs are considered as highly efficient as larvicidal agents when their LC_{50} on larvae is below 50 ppm (Table 6.1). In that case, they own potential to be incorporated in larvicidal formulations (Pavela 2015).

Larvicidal activity of EOs as well as their adulticidal effects is generally provided via contact toxicity (Kumar et al. 2011). Generally, the larvicidal activity of EOs is strongly correlated with the lipophilicity of their constituents. Indeed, the presence in the compound structure of substituents increasing the overall lipophilicity of the molecule results in an enhanced mosquitocidal activity (Samarasekera et al. 2008). In fact, a higher lipophilicity makes an EO more absorbable in larvae midgut (Rey et al. 1999). Thus, the difference in larvicidal activity between different EOs is linked to lipophilicity and diffusion capability of their constituents which in turn depend on difference in their chemical structures. Actually, the presence of oxygenated groups among EO constituents can enhance the overall larvicidal activity. In fact, it has been reported that oxygenated monoterpenes usually display a higher activity than nonoxygenated ones and that, among the former, the activity decrease in the order: phenols > alcohols > aldehydes > ketones, etc. (Pavela 2008). Furthermore, some EOs rich in sesquiterpenes have shown potent larvicidal activity (Pavela 2015). Overall, it has been reported that the presence of hydrocarbon chains linked to a phenyl ring as well as exocyclic double bonds play a key role in the improvement of the larvicidal activity (Benelli et al. 2017a; Pavela 2015). Regarding the latter point, it has been reported that hydrogenation of double bonds in these molecules may restrict the passage throughout the insect cuticle leading to a decrease in biocidal effects (Lomonaco et al. 2009). In addition, the presence of bulky functional groups, such as acyl and aryl ones, tends to increase the EO mosquitocidal activity compared with smaller groups (e.g. acetates) (Kumar et al. 2011).

EO constituents are also capable to deactivate proteins and inhibit enzymes in insects (Ryan and Byrne 1988). Notable larvicidal activity (LC_{50} <50 ppm) was observed for several EO components, namely thymol, carvacrol, (*E*)-anethole, myristicin, dill apiole, *p*-cymene, limonene, β-ocimene and γ-terpinene (Table 6.2) (Govindarajan et al. 2016a; Pavela and Benelli 2016; Pavela et al. 2017; Benelli et al. 2017a). Often, the larvicidal activity of EOs can be enhanced by preparing binary mixtures. For instance, binary mixtures made up of EOs from *Trachyspermum*

Table 6.1 Insecticidal activity of plant-borne EOs, in terms of LC_{50}/LD_{50}, repellency and ovicidal effects, against *Anopheles*, *Aedes* and *Culex* mosquitoes

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm)	Reference
Apiaceae	*Coriandrum sativum* L.	Schizocarps	Linalool	*Aedes albopictus*	Repellent	RD_{50} = 0.0001565 µl cm^{-2}	Benelli et al. (2013)
	Daucus carota L.	Seeds	Carotol	*Culex pipiens pallens*	Larvicidal	53% at 12 ppm	Park and Park (2012)
	Foeniculum vulgare Mill.	Aerial parts Schizocarps	(*E*)-Anethole, limonene, fenchone Methyl chavicol, fenchone	*Aedes aegypti* *Anopheles atroparvus*	Larvicidal Larvicidal	23.3–28.2 46.1	Rocha et al. (2015) Sousa et al. (2015)
	Helosciadium nodiflorum (L.) Koch	Aerial parts	Limonene, myristicin	*Culex quinquefasciatus*	Larvicidal	20.6	Benelli et al. (2017a)
	Pimpinella anisum L.	Schizocarps	(*E*)-Anethole	*Culex quinquefasciatus* *Culex pipiens* *Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	Larvicidal Larvicidal Ovicidal	25.9 <18 15.8–16.6	Benelli et al. (2017a) Kimbaris et al. (2012) Prajapati et al. (2005)
	Smyrnium olusatrum L.	Inflorescences	Isofuranodiene, germacrone	*Culex quinquefasciatus*	Larvicidal	17.5	Benelli et al. (2017a)
	Trachyspermum ammi (L.) Sprague	Schizocarps	Thymol	*Culex quinquefasciatus*	Larvicidal Adulticidal	17.6 µg cm^{-2} 26.5 µg cm^{-2}	Benelli et al. (2017a)
				Anopheles stephensi	Repellent Ovicidal	25.0[a] 49	Pandey et al. (2009)
			Thymol, γ-terpinene, *p*-cymene	*Aedes aegypti*	Larvicidal	80% mortality at 50 ppm	Seo et al. (2012)

(continued)

Table 6.1 (continued)

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm)	Reference
Asteraceae	*Achillea vermicularis* Trin.	Inflorescences	(*E*)-β-Damascenone	*Anopheles stephensi*	Repellent	50% at 5.67 μL cm^{-2}	Pirmohammadi et al. (2016)
	Artemisia absinthium L.	Aerial parts	(*E*)-β-Farnesene, (*Z*)-en-yn-dicycloether, (*Z*)-β-ocimene	*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	Larvicidal	41.9 46.3 50.6	Govindarajan and Benelli (2016b)
	Artemisia gilvescens Miq.	Aerial parts	Camphor, 1,8-cineole, terpine-4-ol	*Anopheles anthropophagus*	Larvicidal	50	Zhu and Tian (2013)
	Artemisia nilagirica (C.B.CLarke) Pamp.	Aerial parts	Not reported	*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	Larvicidal	30.5 55.0 62.3	Prashant et al. (2006)
	Tagetes erecta L.	Leaves	(*E*)-Caryophyllene, caryophyllene oxide	*Aedes aegypti*	Repellent	>60% at 10 μg cm^{-2}	Ali et al. (2016)
			Limonene, terpinolene, (*Z*)-β-ocimene	*Aedes aegypti* *Anopheles stephensi* *Culex quinquefasciatus*	Larvicidal	13.6 12.1 22.3	Dharmagadda et al. (2005)
	Tagetes minuta L.	Aerial parts	(*E*)-β-Ocimene, verbenone, limonene	*Anopheles gambiae*	Larvicidal	1.5	Kyarimpa et al. (2014)
Geraniaceae	*Pelargonium roseum* Willd.	Leaves	Citronellol, geraniol	*Culex pipiens*	Larvicidal	5.5	Tabari et al. (2017b)
					Ovicidal	0.45	Tabari et al. (2017b)

Lamiaceae	*Mentha longifolia* (L.) L.	Leaves	Piperitenone oxide	*Culex quinquefasciatus*	Larvicidal	17	Pavela et al. (2014)
	Mentha pulegium L.	Leaves	Pulegone, *iso*-menthone	*Culex pipiens* biotype *molestus*	Larvicidal	47.0	Michaelakis et al. (2011)
	Mentha spicata L.	Leaves	Carvone, *cis*-carveol, limonene	*Anopheles stephensi*	Larvicidal	49.7	Govindarajan et al. (2012)
	Mentha spicata subsp. *condensata* (Briq.) Greuter & Burdet	Leaves	Piperitenone, piperitenone oxide, pulegone	*Culex pipiens* biotype *molestus*	Larvicidal	39	Traboulsi et al. (2002)
	Mentha suaveolens Ehrh.	Leaves	Piperitenone oxide	*Culex pipiens*	Larvicidal	47.9	Koliopoulos et al. (2010)
			Piperitenone oxide, carvone	*Culex quinquefasciatus*	Larvicidal	17	Pavela et al. (2014)
	Mentha x *piperita* L.	Leaves	Menthol	*Anopheles annularis* *Anopheles culicifacies* *Culex quinquefasciatus*	Repellent	100% at 3 mL m^{-2} 92% at 3 mL m^{-2} 84% at 3 mL m^{-2}	Ansari et al. (2000)
			Menthol	*Aedes aegypti*	Larvicidal	47.2	Amer and Mehlhorn (2006)
			Menthone, menthol	*Culex pipiens* biotype *molestus*	Larvicidal	40.3	Michaelakis et al. (2011)
	Mentha × *villosa* Huds.	Leaves	Rotundifolone, limonene	*Aedes aegypti*	Larvicidal	45.0	Lima et al. (2014)

(continued)

Table 6.1 (continued)

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm))	Reference
	Ocimum basilicum L.	Leaves	Linalool, methyl eugenol	*Culex tritaeniorhynchus* *Aedes albopictus* *Anopheles subpictus*	Larvicidal	14.0 12.0 9.8	Govindarajan et al. (2013)
			Not reported	*Culex quinquefasciatus*	Larvicidal	40.0	Rajamma et al. (2011)
				Aedes aegypti *Anopheles dirus* *Culex quinquefasciatus*	Ovicidal	<1.9 (EC_{50}, %) <1.4 <0.5	Phasomkusolsil and Soonwera (2012)
				Aedes aegypti *Anopheles dirus* *Culex quinquefasciatus*	Repellent	79.4% at 10% 81.6% at 10% 100% at 10%	Phasomkusolsil and Soonwera (2012)
	Ocimum carnosum (Spreng.) Link & Otto ex Benth.	Leaves	Methyl chavicol, (*E*)-anethole	*Anopheles braziliensis*	Repellent	89% at 10% (v/v) cm^{-2}	de Paula et al. (2003)
	Ocimum gratissimum L.	Leaves	Not reported	*Culex quinquefasciatus*	Larvicidal	42.9	Rajamma et al. (2011)
				Anopheles arabiensis *Culex quinquefasciatus*	Repellent	92% 88.7%	Kweka et al. (2008)
	Ocimum kilimandscharicum Gürke	Leaves	Not reported	*Anopheles arabiensis* *Culex quinquefasciatus*	Repellent	89.8% 90.5%	Kweka et al. (2008)

	Ocimum tenuiflorum L.	Aerial parts	Not reported	*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	Larvicidal	53.7 45.7 25.5	Prashant et al. (2006)
		Leaves		*Culex quinquefasciatus*	Larvicidal	39.3	Rajamma et al. (2011)
	Origanum onites L.	Aerial parts	Carvacrol	*Culex pipiens*	Larvicidal	24.8	Cetin and Yanikoglu (2006)
	Origanum scabrum Boiss. & Heldr.	Aerial parts	Carvacrol, thymol	*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus* *Culex tritaeniorhynchus*	Repellent	100% at 5 mg cm^{-2}	Govindarajan et al. (2016b)
	Origanum syriacum L.	Leaves	Carvacrol, thymol	*Culex pipiens* biotype *molestus*	Larvicidal	36	Traboulsi et al. (2002)
	Pogostemon cablin (Blanco) Benth.	Whole plants	Patchoulol	*Culex pipiens pallens*	Larvicidal	53% at 25 ppm	Park and Park (2012)
	Satureja bachtiarica Bunge	Aerial parts	Carvacrol, *p*-cymene, borneol	*Anopheles stephensi* *Culex quinquefasciatus*	Larvicidal	24.3 45.0	Soleimani-Ahmadi et al. (2017)
	Satureja hortensis L.	Aerial parts	β-Oplopenone, *trans*-carvone oxide	*Anopheles stephensi*	Repellent	50% at 5.63 μL cm^{-2}	Pirmohammadi et al. (2016)
	Satureja montana L.	Aerial parts	Carvacrol, γ-terpinene, *p*-cymene	*Culex pipiens* biotype *molestus*	Larvicidal	37.7	Michaelakis et al. (2007)
	Satureja montana L. subsp. *montana*	Aerial parts	Carvacrol, *p*-cymene, thymol	*Culex quinquefasciatus*	Larvicidal	25.6	Benelli et al. (2017b)

(continued)

Table 6.1 (continued)

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm))	Reference
	Satureja thymbra L.	Aerial parts	Thymol, γ-terpinene, *p*-cymene	*Culex pipiens* biotype *molestus*	Larvicidal	44.5	Michaelakis et al. (2007)
	Thymus leucospermus Hartvig	Aerial parts	*p*-Cymene	*Culex pipiens* biotype *molestus*	Larvicidal Repellent	34.3 77.8% at 1 mg cm^2	Pitarokili et al. (2011)
	Thymus teucrioides subsp. *candilicus* (Beauverd) Hartvig	Aerial parts	*p*-Cymene, γ-terpinene, thymol	*Culex pipiens* biotype *molestus*	Larvicidal Repellent	23.2 78.1% 1 mg cm^{-2}	Pitarokili et al. (2011)
Lauraceae	*Cinnamomum osmophloeum* Kaneh	Leaves	Cinnamaldehyde, cinnamyl acetate	*Anopheles gambiae*	Larvicidal	22.2–58.2 11.0–63.6[c]	Mdoe et al. (2014)
			Cinnamaldehyde	*Anopheles albopictus*	Larvicidal	40.8	Cheng et al. (2009a)
			Cinnamaldehyde, cinnamyl acetate	*Anopheles albopictus*	Larvicidal	46.5	Cheng et al. (2009a)
	Cinnamomum verum J. Presl	Bark	Cinnamaldehyde	*Anopheles tessellatus* *Culex quinquefasciatus* *Aedes aegypti*	Larvicidal	0.3 0.7 2.3	Samarasekera et al. (2005)
		Leaves	Eugenol	*Anopheles tessellatus* *Culex quinquefasciatus* *Aedes aegypti*	Larvicidal	1.0 2.1 1.6	Samarasekera et al. (2005)
	Laurus nobilis L.	Aerial parts	1,8-Cineole, α-terpinyl acetate	*Anopheles stephensi* *Culex pipiens*	Larvicidal	14.9 16.5	Mohammadreza (2010)
				Aedes aegypti	Repellent	40% at 10 μg/cm^2	Tabanca et al. (2013)
	Umbellularia californica (Hook. & Arn.) Nutt.	Leaves	Umbellulone, 1,8-cineole	*Aedes aegypti*	Repellent	50% at 10 μg/cm^2	Tabanca et al. (2013)

Myrtaceae	*Eucalyptus camaldulensis* Dehnh.	Leaves	α-Pinene, α-phellandrene, *p*-cymene	*Aedes aegypti* *Aedes albopictus*	Larvicidal	31 55	Cheng et al. (2009a)
	Corymbia citriodora (Hook.) K.D. Hill & L.A.S. Johnson	Leaves	Not reported	*Anopheles dirus* *Culex quinquefasciatus*	Ovicidal	6.8 (EC_{50}, %) 0.5	Phasomkusolsil and Soonwera (2012)
			Citronellal, citronellol, citronellyl acetate	*Anopheles gambiae*	Larvicidal	33.7	Bossou et al. (2013)
			Not reported	*Anopheles arabiensis*	Repellent	90% at 10% (w/w)	Solomon et al. (2012)
	Eucalyptus deglupta Blume	Leaves	α-Phellandrene, nerolidol	*Culex quinquefasciatus*	Repellent	94% at 8%	Pujiarti and Fentiyanti (2017)
	Eucalyptus grandis W. Hill	Leaves	α-Pinene, 1,8-cineole	*Aedes aegypti*	Larvicidal	32.4	Lucia et al. (2007)
	Eucalyptus nitens (H. Deane & Maiden) Maiden	Leaves	1,8-Cineole, *p*-cymene, flavesone	*Aedes albopictus*	Larvicidal	28.2	Costa et al. (2017)
	Eucalyptus tereticornis Sm.	Leaves	β-Pinene, α-pinene, limonene	*Culex quinquefasciatus*	Repellent	92% at 8%	Pujiarti and Fentiyanti (2017)
			p-Cymene, spathulenol, caryophyllene oxide	*Anopheles gambiae*	Larvicidal	28.0	Bossou et al. (2013)
			Not reported	*Anopheles stephensi*	Larvicidal	18.3–23.8	Senthil Nathan (2007)
	Melaleuca ericifolia Sm.	Leaves	Not reported	*Aedes aegypti*	Repellent	98% at 4% (w/w)	Greive et al. (2010)
	Melaleuca quinquenervia (Cav.) S.T. Blake	Leaves	1,8-Cineole, α-pinene, β-pinene	*Aedes aegypti* *Aedes albopictus* *Culex quinquefasciatus*	Larvicidal	47 49 21	Leyva et al. (2016)
	Myrtus communis L.	Leaves	1,8-Cineole, α-pinene, linalool	*Culex pipiens* biotype *molestus*	Larvicidal	16	Traboulsi et al. (2002)

(continued)

Table 6.1 (continued)

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm))	Reference
	Syzygium aromaticum (L.) Merr. & L.M. Perry	Buds	Eugenol	*Aedes aegypti* *Culex quinquefasciatus*	Larvicidal	21 15	Costa et al. (2005)
			Not reported	*Culex quinquefasciatus*	Repellent	76 and 58% escape response at 2.5% (w/v) following contact and non-contact	Suwansirisilp et al. (2013)
			Not reported	*Aedes aegypti* *Anopheles dirus* *Culex quinquefasciatus*	Ovicidal	1.9 (EC_{50}, %) 4.6 <0.5	Phasomkusolsil and Soonwera (2012)
	Syzygium lanceolatum (Lam.) Wight & Arn.	Leaves	Phenyl propanal, α-humulene, (*E*)-caryophyllene	*Anopheles stephensi* *Aedes aegypti*	Larvicidal	51.2 55.1	Benelli et al. (2016b)
Poaceae	*Cymbopogon citratus* (DC.) Stapf	Leaves	Myrcene, neral, geranial	*Anopheles funestus*	Larvicidal	34.6–35.5	Ntonga et al. (2014)
			Geranial, neral, myrcene	*Anopheles gambiae*	Larvicidal	7.7	Bossou et al. (2013)
			Not reported	*Aedes aegypti* *Anopheles dirus* *Culex quinquefasciatus*	Repellent	ED_{50}<0.045 mg cm^{-2}	Phasomkusolsil and Soonwera (2011)

			Not reported	*Culex quinquefasciatus*	Repellent	87 and 83% escape response at 10% (w/v) following contact and non-contact	Suwansirisilp et al. (2013)
			Not reported	*Aedes aegypti* *Anopheles dirus* *Culex quinquefasciatus*	Ovicidal	<1.9 (EC_{50}, %) 4.8 <0.5	Phasomkusolsil and Soonwera (2012)
	Cymbopogon nardus (L.) Rendle	Leaves	Not reported	*Anopheles arabiensis*	Repellent	90% at 10% (w/w)	Solomon et al. (2012)
Pinaceae	*Cedrus libani* A. Rich.	Seeds	Not reported	*Culex pipiens*	Larvicidal	47.8	Cetin et al. (2009)
	Pinus nigra J.F. Arnold var. *italica*	Leaves	α-Pinene, germacrene D, (*E*)-caryophyllene	*Culex quinquefasciatus*	Larvicidal	49.8	Benelli et al. (2017b)
	Pinus tropicalis Morelet	Resin	α-Pinene, β-pinene	*Aedes aegypti*	Larvicidal	21.4	Leyva et al. (2009)
Rutaceae	*Citrus* x *aurantium* L.	Fruit peel	Limonene	*Anopheles stephensi*	Larvicidal	31.2	Sanei-Dehkordi et al. (2016)
				Culex pipiens biotype *molestus*	Larvicidal	39.8–52.0	Michaelakis et al. (2009)
		Aerial parts	Limonene	*Anopheles labranchiae*	Larvicidal	22.6	El-Akhal et al. (2015)
	Citrus limon (L.) Osbeck	Fruit peel	Limonene, β-pinene, γ-terpinene	*Culex pipiens* biotype *molestus*	Larvicidal	30.1	Michaelakis et al. (2009)
	Citrus paradisi Macfad.	Fruit peel	Not reported	*Anopheles stephensi*	Larvicidal	35.7	Sanei-Dehkordi et al. (2016)

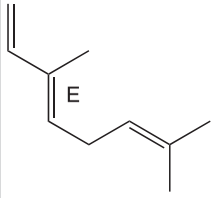
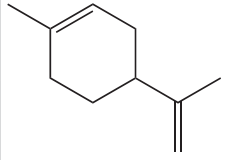
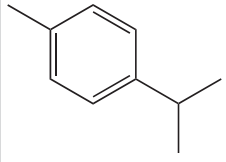
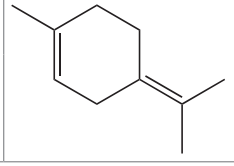
(continued)

Table 6.1 (continued)

Family	Species	Part used	Major EO compounds	Mosquito target	Effect	LC_{50}/LD_{50} (ppm))	Reference
	Citrus sinensis (L.) Osbeck	Fruit peel	Limonene	*Aedes aegypti*	Larvicidal	11.9–16.3	Araujo (2016)
			Not reported	*Aedes aegypti*	Repellent	55.3% at 10%	Phasomkusolsil and Soonwera (2012)
			Not reported	*Aedes aegypti* *Anopheles dirus* *Culex quinquefasciatus*	Ovicidal	4.5 (EC_{50}, %) 5.1 <0.5	Phasomkusolsil and Soonwera (2012)
Verbenaceae	*Lippia multiflora* Moldenke	Leaves	Thymol, *p*-cymene, thymol acetate	*Anopheles gambiae*	Ovicidal	17.1	Bassolé et al. (2003)
Zingiberaceae	*Zingiber officinale* Roscoe	Rhizome	Not reported	*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	Ovicidal	32.2[d] 52.7[d] 53.4[d]	Prajapati et al. (2005)

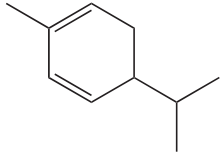
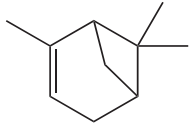
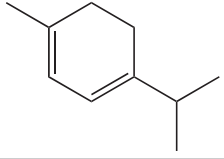
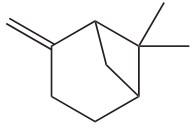
[a]RD_{50} value, expressed in mg mat^{-1}, has been reported; mat = cardboard sheet
[c]Values obtained in semifield conditions
[d]LC_{95} values has been reported

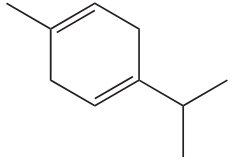
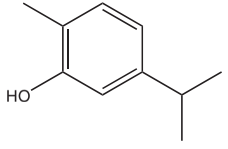
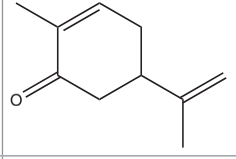
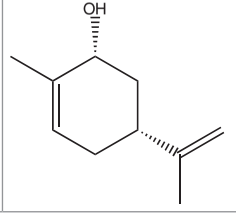
Table 6.2 Larvicidal activity of various EO components against *Anopheles*, *Aedes* and *Culex* mosquitoes

Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
Monoterpene hydrocarbons	(*E*)-β-Ocimene		*Anopheles stephensi* *Anopheles subpictus* *Aedes aegypti* *Aedes albopictus* *Culex quinquefasciatus* *Culex tritaeniorhynchus*	25.8 28.4 31.5 20.9 33.7 37.1	Govindarajan and Benelli (2016b)
	Limonene		*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	8.8 12.0 14.1	Govindarajan et al. (2012)
			Aedes aegypti *Aedes albopictus*	18 32.7	Cheng et al. (2009a, b)
	p-Cymene		*Aedes aegypti* *Aedes albopictus*	19.2 46.7	Cheng et al. (2009a)
			Culex quinquefasciatus	20.6	Pavela et al. (2017)
	Terpinolene		*Aedes aegypti* *Aedes albopictus*	28.4 35.6	Cheng et al. (2009a)
			Culex quinquefasciatus	25.7	Pavela et al. (2018)

(continued)

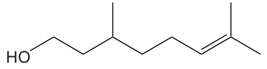
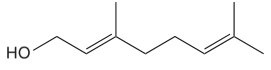
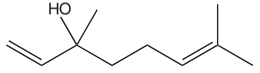
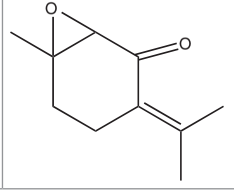
Table 6.2 (continued)

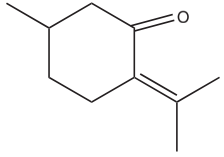
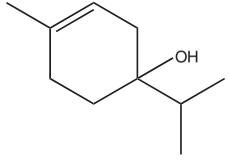
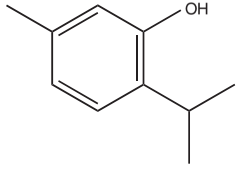
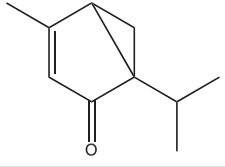
Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
	α-Phellandrene		*Aedes aegypti* *Aedes albopictus*	16.6 39.9	Cheng et al. (2009a, b)
	α-Pinene		*Aedes aegypti*	15.4	Lucia et al. (2007)
			Culex pipiens biotype *molestus*	47–49	Traboulsi et al. (2002)
	α-Terpinene		*Aedes aegypti* *Aedes albopictus*	14.7 25.2	Cheng et al. (2009a)
	β-Pinene		*Aedes aegypti*	12.1	Lucia et al. (2007)
			Anopheles stephensi *Culex quinquefasciatus*	23.2 32.2	Govindarajan (2010)
			Aedes albopictus	42.4–47.3	Giatropoulos et al. (2012)
			Culex pipiens biotype *molestus*	36.5	Michaelakis et al. (2009)

	γ-Terpinene		*Culex quinquefasciatus*	16.7	Pavela et al. (2017)
			Aedes aegypti *Aedes albopictus*	30.7 29.8	Cheng et al. (2009a)
Oxygenated monoterpenes	Carvacrol		*Culex pipiens* biotype *molestus*	37.6	Traboulsi et al. (2002)
			Anopheles stephensi *Anopheles subpictus* *Culex quinquefasciatus* *Culex tritaeniorhynchus*	21.2 24.1 26.1 28.0	Govindarajan et al. (2016a)
	Carvone		*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	19.3 23.7 25.5	Govindarajan et al. (2012)
	cis-Carveol		*Anopheles stephensi* *Aedes aegypti* *Culex quinquefasciatus*	28.5 32.9 35.2	Govindarajan et al. (2012)

(continued)

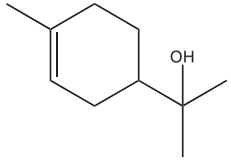
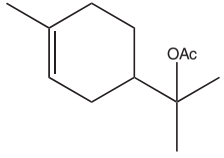
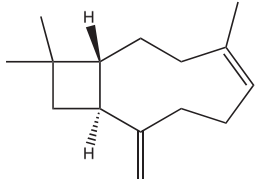
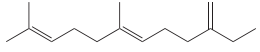
Table 6.2 (continued)

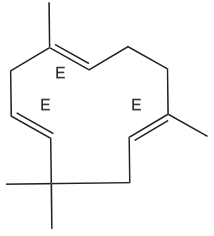
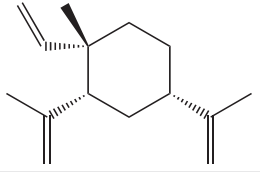
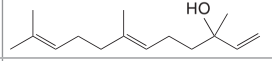
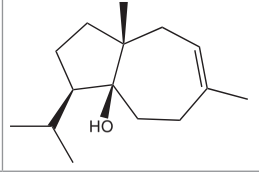
Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
	Citronellol		*Aedes aegypti*	49.9	Ali et al. (2013)
			Culex pipiens	7.6	Tabari et al. (2017b)
	Geraniol		*Aedes aegypti*	49.3	Ali et al. (2013)
			Culex pipiens	6.1	Tabari et al. (2017b)
	Linalool		*Culex pipiens*	14.9	Tabari et al. (2017b)
	Piperitenone oxide		*Culex pipiens* biotype *molestus*	10.0	Koliopoulos et al. (2010)

	Pulegone		*Culex pipiens* biotype *molestus*	27.2	Michaelakis et al. (2011)
	Terpinen-4-ol		*Anopheles stephensi* *Anopheles subpictus*	43.3 47.7	Govindarajan et al. (2016a)
	Thymol		*Anopheles stephensi*	48.9	Pandey et al. (2009)
			Aedes aegypti	17.5	Tabanca et al. (2013)
			Culex pipiens biotype *molestus*	36	Traboulsi et al. (2002)
			Culex quinquefasciatus	15.1	Pavela et al. (2018)
	Umbellulone		*Aedes aegypti*	32.3	Tabanca et al. (2013)

(continued)

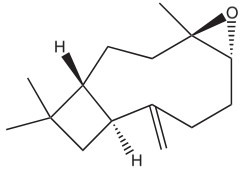
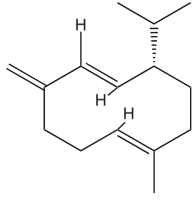
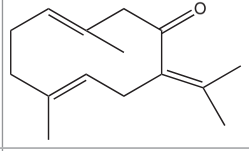
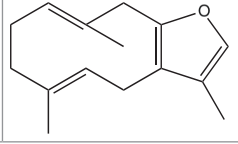
Table 6.2 (continued)

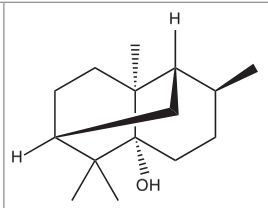
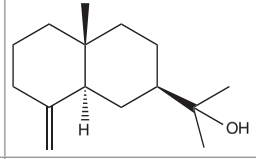
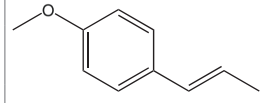
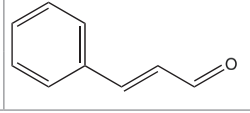
Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
	α-Terpineol		*Culex pipiens* biotype *molestus*	16	Traboulsi et al. (2002)
	α-Terpinyl acetate		*Culex pipiens*	23.0	Kimbaris et al. (2012)
Sesquiterpene hydrocarbons	(*E*)-Caryophyllene		*Anopheles subpictus* *Aedes albopictus* *Culex tritaeniorhynchus*	41.7 44.8 48.2	Govindarajan et al. (2016c)
	(*E*)-β-Farnesene		*Anopheles stephensi* *Anopheles subpictus* *Aedes aegypti* *Aedes albopictus* *Culex quinquefasciatus* *Culex tritaeniorhynchus*	8.1 8.8 9.7 10.2 11.4 13.0	Govindarajan and Benelli (2016b)

	α-Humulene		*Anopheles subpictus* *Aedes albopictus* *Culex quinquefasciatus*	6.2 6.9 7.4	Govindarajan and Benelli (2016a)
	β-Elemene		*Anopheles subpictus* *Aedes albopictus* *Culex quinquefasciatus*	10.3 11.2 12.0	Govindarajan and Benelli (2016a)
Oxygenated sesquiterpenes	(*E*)-Nerolidol		*Aedes aegypti*	13.4	Ali et al. (2013)
	Carotol		*Culex pipiens*	29.4	Park and Park (2012)

(continued)

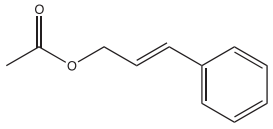
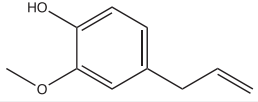
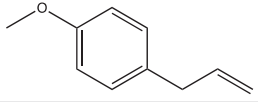
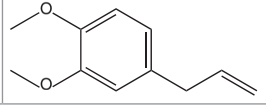
Table 6.2 (continued)

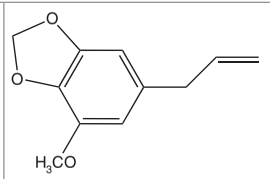
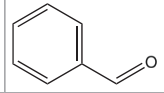
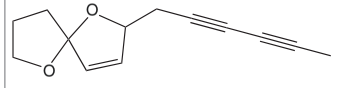
Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
	Caryophyllene oxide		*Anopheles anthropophagus*	49.5	Zhu and Tian (2013)
	Germacrene D		*Anopheles anthropophagus*	49.8	Zhu and Tian (2013)
	Germacrone		*Culex quinquefasciatus*	18.6	Benelli et al. (2017a)
	Isofuranodiene		*Culex quinquefasciatus*	33.7	Benelli et al. (2017a)

	Patchouli alcohol		*Culex pipiens*	47.2	Park and Park (2012)
	β-Eudesmol		*Culex pipiens*	27.7	Park and Park (2012)
Phenylpropanoids	(*E*)-Anethole		*Culex quinquefasciatus*	15.3–19.8	Pavela (2014a, b)
			Culex pipiens	16.6	Kimbaris et al. (2012)
			Aedes aegypti	42	Cheng et al. (2004)
			Anopheles atroparvus	15.7	Sousa et al. (2015)
	Cinnamaldehyde		*Aedes aegypti*	29	Cheng et al. (2004)
			Aedes albopictus	48.1	Cheng et al. (2009b)

(continued)

Table 6.2 (continued)

Chemical group	Name	Chemical structure	Mosquito target	LC_{50}/LD_{50} (ppm)	Reference
	Cinnamyl acetate		*Aedes aegypti*	33	Cheng et al. (2004)
	Eugenol		*Culex pipiens*	18.3	Kimbaris et al. (2012)
			Aedes aegypti	33	Cheng et al. (2004)
	Methyl chavicol		*Aedes aegypti*	46.4	Rocha et al. (2015)
	Methyl eugenol		*Aedes aegypti*	36.5	Tabanca et al. (2013)

	Myristicin		*Culex quinquefasciatus*	16.3	Pavela et al. (2017)
Others	Benzaldehyde		*Aedes albopictus*	47.0	Cheng et al. (2009b)
	(*Z*)-en-yn-Dicycloether		*Anopheles stephensi* *Anopheles subpictus* *Aedes aegypti* *Aedes albopictus* *Culex quinquefasciatus* *Culex tritaeniorhynchus*	16.2 17.7 19.8 21.0 23.5 25.9	Govindarajan and Benelli (2016b)

ammi (L.) Sprague plus *Pimpinella anisum* L. and *Smyrnium olusatrum* L. plus *P. anisum* in different ratios have provided synergistic effects. These mixtures showed larvicidal effects (LC_{50} <20 ppm) comparable to those of commercial products with short lethal times (Benelli et al. 2017a).

6.4.2 Growth and Reproduction Inhibition

The transition from the larval stage to adulthood is a critical point for the establishment of a mosquito population density able to vector pathogens in a certain area. This transition may be disrupted or prevented through the application of EO-based products that alter physiological processes involved in metamorphosis leading to sterility, malformation and death (Kumar et al. 2011). EOs may be involved in biochemical processes influencing the endocrinologic balance of insects (Rattan 2010). Furthermore, EOs interfere with fecundity and fertility of female mosquitoes by reducing egg hatchability and oviposition (Pavela 2015; Benelli 2015b; Autran et al. 2009). Their vapours diffuse into eggs interrupting embryonic development (Papachristos and Stamopoulos 2004). For instance, application of *Mentha* x *piperita* L. EO resulted in a reduction of the reproductive potential in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* (Ansari et al. 2000), whereas that of *M. spicata* L. affected *An. stephensi* fecundity and fertility (Tripathi et al. 2004). In addition, EOs can inhibit or reduce food intake in several mosquito vectors (Pavela and Benelli 2016).

6.4.3 Repellency

EOs are good candidates as repellent agents since they are capable to deter mosquitoes from flying to and landing on skin and sucking blood by acting locally or at a distance (Blackwell et al. 2003; Nerio et al. 2010). This property is related to the chemico-physical characteristics of EO constituents such as their high volatility. For long time, the only repellent available on the market was DEET (*N*,*N*-diethyl-*m*-toluamide), but in the last decades, several EO-based repellents for applications to human skin have been introduced. One of the first EOs used for this scope was that of lemongrass (*Cymbopogon citratus* (DC.) Stapf) often mixed with that of peppermint (*M.* x *piperita*). Generally, the repellency of EO constituents increases as the number of methyl groups in the side chain of the molecule increases (Aggarwal et al. 2001). As an example, peppermint EO protected from *An. annularis*, *An. culicifacies*, *Ae. albopictus* and *Cx. quinquefasciatus* with efficacy comparable to that of Mylol, a commercial mosquito repellent (Ansari et al. 2000; Yang and Ma 2005). *M. spicata* L. EO provided protection against flights of *An. stephensi* (Table 6.1) (Tripathi et al. 2004). Repellent effects may be given by either major or minor constituents of EOs (Samarasekera et al. 2008).

6.4.4 Antifeedant

Antifeedants can be described as allomone substances which inhibit feeding and do not kill the pest directly but rather limit its development potential considerably and act as phagodeterrent or phagorepellent agents. Their great advantage is their selective action against parasites and pest predators, as well as pollinators. Plant substances acting as antifeedants are found in all the compound groups of secondary plant metabolism. However, the most effective insect feeding inhibitors come from terpenoids, alkaloids, saponins and polyphenols (Koul 2005). Several EOs have shown antifeedant activity against mosquito vectors, for instance, *Eucalyptus tereticornis* Sm. against *An. stephensi*; *Chloroxylon swietenia* DC. against *An. gambiae*, *Cx quinquefasciatus* and *Ae. aegypti*; and *Calocedrus decurrens* (Torr.) Florin and *Juniperus occidentalis* Hook. against *A. aegypti* (Rattan 2010).

6.4.5 Neutralization of Insect Defence

In order to defend themselves from exogenous substances, insects have evolved different systems such as cytochrome P450, glutathione-S-transferases and esterases. These systems are placed in the midgut of insect, and their primary function is to eliminate the ingested or adsorbed toxic compounds allowing detoxification of the body from exogenous substances penetrated from cuticle or trachea (Schuler 1996). Among the EO components acting on the insect defence system, phenylpropanoids such as eugenol, myristicin and dill apiole play an important role (Table 6.2) (Afshar et al. 2017). By the way, more research on these targets are encouraged in the next years.

6.4.6 Neurotoxicity

Many EOs exert relevant neurotoxic effects in insects causing a state of hyperactivity followed by hyper-excitation resulting in knock-down and immobilization (Rattan 2010; Enan 2001). The cholinergic system is an important target of many EO constituents like monoterpenes (e.g. limonene, terpinolene, β-pinene) which interfere with nerve conduction and coordination of the neuromuscular system causing alteration of insect behaviour, lack of coordination and possible death (Choi et al. 2006; Pavela 2015; Felipe et al. 2008; Ryan and Byrne 1988; Fournier and Mutero 1994). Some EOs (e.g. tea tree, lavender, ginger) and their main constituents (Tabari et al. 2017a) were proven as reversible competitive inhibitors of acetylcholinesterase (AChE) enzymes (Ryan and Byrne 1988; Keane and Ryan 1999; Mills et al. 2004).

Another possible target is given by the GABA receptors also known as GABA-gated chloride channels (Rattan 2010). Blockage of these channels results in a hyper-excitation state, convulsions and death (Bloomquist 2003). GABA receptors are also involved in the stimulation of feeding and taste. Therefore, GABA inhibitors can also be considered as good antifeedant agents (Mullin et al. 1994). Monoterpenes such as thujone and thymol are important modulators of GABA-gated chloride channels in insects (Hold et al. 2000; Priestley et al. 2003). Among sesquiterpenes, the tricyclic silphinenes which are restricted to few essential oils (Pavela et al. 2016) are recognized as significant GABA inhibitors (Bloomquist et al. 2008; Rattan 2010).

Furthermore, modulation of octopamine receptors can be another hallmark of EOs' mode of action. Octopamine receptors are coupled to G proteins (Enan 2005) and bind to the neurotransmitter and neuromodulator octopamine, a biogenic amine occurring in insects that plays important physiological functions equivalent to those of norepinephrine in vertebrates (Enan 2001). Octopamine receptors are often antagonized by EO components like β-pinene, limonene, terpinolene, thymol and eugenol that cause alteration of insect behaviour, e.g. increase of heartbeat and cAMP production (Enan 2005; Tak et al. 2015; Rattan 2010).

Overall, though many studies have been conducted on the toxic effects of EOs on target organisms, research concerning the effects of sublethal concentrations of EOs on target and nontarget organisms (e.g. aquatic plankton, beneficial arthropods, small vertebrates) as well as on the mechanisms of action and relationships among EO bioactive compounds should be boosted in the next years.

6.5 Essential Oil-Based Marketed Products

The market of EOs as raw materials for the manufacture of flavours and fragrance is expected to grow by 5% per year (CBI 2009b). Interesting perspectives are also opening in the global market of insecticides where the public opinion's preference is moving towards more healthy and eco-friendly products. Although plant EOs have been fully explored and scientifically validated for efficacy against mosquitoes and agricultural pests, only a few products are commercially available to date.

Overall, most of EOs are currently used as repellent agents against mosquitoes (Benelli et al. 2013; Giatropoulos et al. 2013) although many of them could be suitable also as ingredients of adulticidal and larvicidal formulations (Pavela et al. 2009; Pavela 2009; Dias and Moraes 2014). The US market was the pioneer in using EOs obtained from thyme, rosemary, clove, peppermint and cinnamon as botanical insecticides and pesticides through the EcoSMART technologies over a decade ago (Isman et al. 2011). In fact, in this country, EO-based insecticides bypass the registration process if the EO comes from a plant used in food and/or beverages. Other important achievements in the development of EO-based insecticides were obtained through the approval of Requiem® (2011) and PREV-Am® (2014), two products based on EOs of *Dysphania ambrosioides* (L.) Mosyakin & Clemants and citrus peel, respectively (Isman 2015). Furthermore, EOs from citronella (*Cymbopogon citratus* (DC.) Stapf), clove (*Syzygium aromaticum* (L.) Merr.

& L.M. Perry) and spearmint (*Mentha spicata* L.) were approved as repellent agents (EU Pesticides Database 2015). Other EO-based products manufactured in countries with leaders in the research on botanical insecticides like India and China are those obtained from *Eucalyptus globulus* Labill. and *Cinnamomum camphora* (L.) J. Presl. (Isman 2015). In Australia, the insecticide/acaricide Eco-oil®, made up of the binary mixture of *Melaleuca alternifolia* (Maiden & Betche) Cheel (tea tree) and *Eucalyptus* EOs, is produced by organic farmers (Isman 2017).

In spite of that, there are still several hurdles to overcome before fully exploiting EOs on an industrial scale. As an example, the regulatory restrictions in the European Union hindered their approval so far. On the other hand, the multinational agrochemical companies introduced on the market a new generation of synthetic insecticides endowed with lower risks for health and environment compared with the previous and older products (Wing et al. 2000; Isman 2015). Other points are the limited number of field trials, the low persistence of effects and the lack of raw material at low costs (Pavela and Benelli 2016). All these factors definitely delayed a fast and wide diffusion and marketing of EO-based formulations. Nevertheless, it has been estimated that botanical insecticides will gain 7% of the global pesticide market by 2025 (Isman 2015). Moreover, the market of synthetic pesticides is expected to decrease by 1.5% per year (Thakore 2006). Regarding the European Union, here the European Food Safety Authority (EFSA) is simplifying the regulatory path for some botanicals by evaluating them as 'low-risk active substances' (LRASs) as reported in the EC Regulation No. 1107/2009. Another element favouring the use of plant EOs as botanical insecticides is the fact that most of their major components are generally recognized as safe (GRAS) by both the US FDA (Food and Drug Administration) and the EPA (Environmental Protection Agency) for use in food and beverages so that their negative impact on human health and environment can be considered as negligible (Miresmailli and Isman 2014).

From a manufacturing perspective, nowadays are considered promising and exploitable on an industrial scale those EOs satisfying the following criteria: (1) availability and cultivation on a large scale of the plant source, (2) high EO yield and (3) low prices of EO (generally correlated with the yield) and raw material from which EOs are obtained. Therefore, agrochemical companies should be oriented towards EOs for which a global production of at least 50 tonnes can be assured (CBI 2009a; Shrinivas and Kudli 2008). Regarding these points, it is worth to note that European countries like France, Italy and Spain own the leaderships in Europe in the production of high-yield EOs thanks to the presence of cultivation, collection and processing procedures carried out by mechanized techniques (CBI 2009a). Thus, the commercial price of EOs shows a wide range of variability, going from cheap EOs obtained on a large scale to very expensive EOs available and marketed in little amounts (CBI 2009a). Overall, the price of EOs should be calculated as five times that of the raw material from which they are obtained (CBI 2009a). On the other hand, developing countries are emerging among the most important producers of EOs owing to the lower labour costs. Overall, the world leadership, in terms of EOs manufacture volume, belongs to the USA (24%) followed by China (20%) and Brazil (8%), whereas the other countries, including European ones, are all by 5% of global volume (Lubbe and Verpoorte 2011). Meanwhile the cultivation of medicinal

and aromatic plants for the production of botanical insecticides appears to be as the most suitable strategy, other options coming from wild-growing plants or cell cultures seem to be devoid of advantages and not pursued.

6.6 Toxicity of EOs

Since many EOs have been used for long times as flavouring and fragrances, we should expect they do not produce significant acute toxicity on mammals. Indeed, many components of EOs used in the food, cosmetic and fragrance industries enjoy the GRAS status released by the FDA and EPA (Miresmailli and Isman 2014). However, it has to be taken into account that some EOs, whether not properly diluted in a formulation, may be irritant to the skin, eyes and mucosae (Isman and Machial 2006).

Generally, ketone-containing EOs are those causing the major toxicity. For instance, pulegone, one of the major constituents of pennyroyal (*Mentha pulegium* L.) EO (Teixeira et al. 2012), is reported as highly toxic to mice showing an LD_{50} of 150 mg/kg (Table 6.3), and pennyroyal EO poisoning has been registered in both humans and animals (Anderson et al. 1996). α-Thujone, one of the main components of wormwood (*Artemisia absinthium* L.) and sage (*Salvia officinalis* L.) EOs, is very toxic to rats showing an LD_{50} of 45 mg/kg (Table 6.3) (Hold et al. 2000).

To have an idea of the relatively safety of some EO constituents, eugenol, the major component of clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) EO, is about 1500 times less toxic than pyrethrum and much less toxic (about 15,000 times) than the synthetic insecticide azinphosmethyl to rainbow trout *Oncorhynchus mykiss* (Walbaum) (Stroh et al. 1998).

Actually, to fulfil the requirements of regulatory agencies, an EO-based formulation should have an LD_{50} above 5 g/kg in rats (Isman and Machial 2006). However, it is important to underline that, whereas data on the acute toxicity of EOs are available in large quantities, information on the chronic and subchronic toxicity, inhalation effects and immunotoxicity is poor.

Overall, most EOs volatilize quickly in the environment leaving no or little residues and allowing immigration or reintroduction of beneficial organisms such as honeybees and other pollinators. Anyway, further research on the effects of EOs on pollinators and natural enemies of target insects under field conditions are urgently needed in the future years.

6.7 Botanical Families Serving as Sources of Mosquitocidal EOs

Higher plants belonging to gymnosperms and angiosperms have evolved specialized secretory tissues where they produce and compartmentalize secondary metabolites including EOs. These structures can be divided into two groups, namely,

Table 6.3 Toxicity, expressed as lethal dose-50 (LD_{50}), of some EOs and their main components[a]

	Animal	Administration	LD_{50} (mg/kg)[a]
Pure compound			
(*E*)-Anethole	Rats	Oral	2090
1,8-Cineole	Rats	Oral	2480
Carvone	Rats	Oral	1640
Cinnamaldehyde	Rats	Oral	1160
Citral	Rats	Oral	4960
Dill apiole	Rats	Oral	1000
Eugenol	Rats	Oral	2680
Limonene	Rats	Oral	4600
Menthol	Rats	Oral	3180
Pulegone	Mice	Intraperitoneal	150
Thujone	Mice	Subcutaneous	87.5
Thymol	Rats	Oral	980
γ-Terpinene	Rats	Oral	1680
EO			
Cinnamomum camphora	Rats	Oral	3730
Citrus sinensis	Rats	Oral	>5000
Coriandrum sativum	Rats	Oral	4130
Cymbopogon citratus	Rats	Oral	>5000
Foeniculum vulgare	Rats	Oral	3120
Lavandula angustifolia	Rats	Oral	4250
Melaleuca alternifolia	Rats	Oral	1900
Ocimum basilicum	Rats	Oral	>5000
Rosmarinus officinalis	Rats	Oral	>5000
Thymus vulgaris	Rats	Oral	2840
Zingiber officinale	Mice	Oral	3400

[a]Sources: Dev and Koul (1997), Food and Agriculture Organization of the United Nations (1999), Koul (2005), Pavela and Benelli (2016), and Isman and Machial (2006)

external and internal ones. The former are of epidermal origin and exude continuously secretions outside the plant (e.g. capitate trichomes) or as a response to touches and shocks and release the material to trap and kill insects (e.g. peltate trichomes of the Lamiaceae, capitate trichomes of cannabis) (Miresmailli and Isman 2014). The latter are of parenchymatic origin and keep the secretion inside the plant tissues. Main examples are represented by idioblasts (e.g. *Laurus nobilis* L.), resin channels (e.g. Pinaceae), ducts and vittae (Apiaceae), translucent glands (*Hypericum* spp.) and oil glands (e.g. Rutaceae).

The presence of the above structures, either external and internal ones, makes plants a rich reservoir of EOs, thus potentially employable as sources of botanical insecticides. The most important families in the angiosperms, in terms of EOs production, are (in alphabetical order): Apiaceae, Asteraceae, Geraniaceae, Lamiaceae, Lauraceae, Poaceae, Myrtaceae, Rutaceae, Verbenaceae and Zingiberaceae.

On the other hand, plants devoid of secretory structures give very low EO yields so that eventual applications in the manufacture of botanical insecticide are not feasible.

In the following section, the main examples of effective EOs (i.e. with LC_{50}/LD_{50}/EC_{50} values below 50 ppm) produced by the most important plant families against *Anopheles*, *Aedes* and *Culex* species will be reported. To the best of our knowledge, until now the scientific research focused mainly on larvicides and repellents, whereas little efforts have been made to identify effective ovicides (Benelli 2015b).

6.7.1 Apiaceae

According to The Plant List (http://www.theplantlist.org), the family of Apiaceae (formerly named Umbelliferae) comprises 418 genera and 3257 species of aromatic herbs distributed mainly in temperate regions in the northern hemisphere. They are endowed with erect, stout, hollow stems, alternate leaves, composite inflorescences (umbels) and dry fruits (schizocarps). All plant organs are rich of internal secretory structures, namely ducts (channel) and vittae (in the fruits) where EOs are stored (Cianfaglione et al. 2017).

6.7.1.1 *Pimpinella anisum* L.

Well-known as aniseed, *P. anisum* is an annual herb widely cultivated in the Mediterranean area and used as flavouring of foodstuffs, liqueurs, confectionery and bakery products (Iannarelli et al. 2017). In the traditional medicine, it has been used to treat gastrointestinal problems, such as galactugoge and expectorant (Andallu and Rajeshwari 2011). The EO is obtained from the fruits (schizocarp) with an average yield in the range 2–6%. The price on the market has been estimated in 7–9 €/kg (Lubbe and Verpoorte 2011). Aniseed EO composition is dominated by the phenylpropanoid (*E*)-anethole, accounting for 75–95% of the total composition. Both aniseed EO and (*E*)-anethole have been reported as potent larvicidal and adulticidal against mosquitoes. When tested against *Cx. quinquefasciatus* third instar larvae, aniseed EO showed an LC_{50} value of 25.9 ppm which was consistent with that of its major component (LC_{50} = 24.8 ppm) (Benelli et al. 2017a). In addition, (*E*)-anethole and aniseed EO have shown synergism when mixed with other substances or EOs (*Trachyspermum ammi* (L.) Sprague and *Smyrnium olusatrum* L.) showing a reduction in the LC_{50} values (<20 ppm) (Benelli et al. 2017a). Furthermore, aniseed EO and (*E*)-anethole were toxic to the West Nile vector *Cx. pipiens* (Kimbaris et al. 2012). Noteworthy, aniseed EO was not toxic to nontarget organisms such as *Daphnia magna* (Straus) at long exposure times and low concentrations (Pavela 2014a).

6.7.1.2 *Trachyspermum ammi* (L.) Sprague

T. ammi, also known as ajwain, is an annual herb occurring in the arid regions of Egypt, Iran, Iraq, Afghanistan, Pakistan and India (Vitali et al. 2016). The fruits (schizocarps) are used as flavourings of foodstuffs and as preservatives; they are also employed in the traditional medicine in the treatment of flatulence, gastrointestinal diseases, abdominal tumours and respiratory problems (Bairwa et al. 2012). The fruits contain 2–5% of EO of pronounced odour, also known as ajwain oil. This EO is characterized by the phenolic thymol (35–60%) and its parent molecules *p*-cymene and γ-terpinene (Zarshenas et al. 2014). Ajwain EO and its main component thymol showed significant larvicidal, oviposition-deterrent, vapour toxicity and repellent effects on *An. stephensi* (Pandey et al. 2009) (Table 6.1). They were both effective as larvicidal against *Cx. quinquefasciatus* with LC_{50} of 18 ppm (Pavela 2015; Benelli et al. 2017a, b). Ajwain EO was toxic also on larvae of *Ae. aegypti* (Table 6.1) (Seo et al. 2012). Besides thymol, also γ-terpinene and *p*-cymene may contribute to the mosquitocidal activity of ajwain EO. Indeed, they were both very toxic on *Cx. quinquefasciatus* (Table 6.2) (Pavela et al. 2017). Ajwain EO showed synergism with that of *P. anisum* (ratio 1:2) against third instar larvae of *Cx. quinquefasciatus* and contact toxicity against adults of the same mosquito (Benelli et al. 2017a, b)

6.7.1.3 *Smyrnium olusatrum* L.

S. olusatrum, also known as Alexanders or wild celery, is an overlooked horticultural crop widely cultivated during the Roman age and then abandoned after the introduction of common celery (*Apium graveolens* L.) (Maggi et al. 2012b). In the past, Alexanders was employed as a vegetable and also as antiscorbutic, diuretic, stomachic, laxative and depurative (Quassinti et al. 2013; Quassinti et al. 2014). All its parts exude a myrrh-scented EO which is dominated by oxygenated sesquiterpenes containing a furan ring among which isofuranodiene is the most abundant compound (Maggi et al. 2012a, b, 2015). The EO hydrodistilled from Alexanders inflorescences exhibited noteworthy larvicidal effects against *Cx. quinquefasciatus* with a LC_{50} of 17.5 ppm (Table 6.1); among its major compounds, germacrone and isofuranodiene showed relevant toxicity on larvae of the same mosquito, with LC_{50} of 18.6 and 33.7 ppm, respectively (Table 6.2) (Benelli et al. 2017a, b).

6.7.2 *Asteraceae*

The Asteraceae family, also known as Compositae due to the characteristic composite inflorescences called capitula, represents the largest group of flowering plants in the angiosperm dicotyledons able to colonize every habitat in the planet. It encompasses 1911 genera and about 32913 species (http://www.theplantlist.org/) with a

cosmopolitan distribution. The Asteraceae are mostly herbs equipped with alternate, simple or pinnatisect leaves. Flowers are small and clustered in apical or axillary capitula; they are inserted on a receptacle and are surrounded by an involucre of bracts; the inner florets (disc florets) are tubular and hermaphrodite, the outer florets (ray florets) are straplike, female or neuter; they are formed by a gamopetalous corolla, five-lobed, and bear five stamens joined into a tube crossed by a bifid stigma. The fruits are cypselas, i.e. achenes accompanied by hairs or bristles of calyx (pappus). The family is divided into two subgroups: i) the Asteroideae (or Tubuliflorae) endowed with capitula formed by tubular and straplike florets, or by all tubular florets, and that produce mostly EOs and bitter compounds, and ii) Cichorioideae (or Liguliflorae) endowed with capitula formed by all straplike florets, which produce latex and phenolic compounds. The most important genera for the production of EOs are *Helichrysum*, *Achillea*, *Artemisia*, *Chamaemelum*, *Matricaria* and *Tagetes*.

6.7.2.1 *Artemisia absinthium* L.

A. absinthium, known as absinth, is an aromatic herb occurring in arid places of Southern Europe and traditionally used as antihelmintic, stomachic, antibacterial, antipyretic, antimalarial, antitumour and hepatoprotective (Amat et al. 2010). Absinth is widely used as flavouring in alcoholic beverages although recently the use of thujone-rich genotypes has been restricted in the European Union due to the high toxicity of thujone (Table 6.3) (Blagojevic et al. 2006). The EO is reported to be antimicrobial, antiprotozoal, fungicidal, acaricidal and insecticidal (Bailen et al. 2013). Nonetheless, the EO chemical composition is quite variable, being subjected to the influence of environmental and genetic factors. As a matter of fact, an absinth chemotype rich in (*E*)-β-farnesene, (Z)-en-yn-dicycloether and (Z)-β-ocimene was highly toxic on larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, exhibiting LC_{50} of 41.9, 46.3 and 50.6 ppm, respectively (Table 6.1) (Govindarajan and Benelli 2016b).

6.7.2.2 *Tagetes minuta* L.

T. minuta also known as African marigold is a weed belonging to the Asteraceae family native to South America but naturalized all over the world (Negahban et al. 2013). It is used industrially as a source of EO and carotenoids (dos Santos et al. 2017). Notably its EO owns potential as a natural preservative agent because of the noteworthy antibacterial activity (Céspedes et al. 2006). In the traditional medicine, *T. minuta* has been used as anthelminthic, antifungal, antiseptic, diuretic, antispasmodic and antitussive. Its EO is also known as insecticidal. In particular, it showed relevant toxicity against larvae of *An. gambiae* showing a LC_{50} of 1.5 ppm (Table 6.1) (Kyarimpa et al. 2014). The EO is made up of (*E*)-β-ocimene, limonene and verbenone with documented mosquitocidal effects (Table 6.2) (Govindarajan and Benelli 2016b; Govindarajan et al. 2012; Cheng et al. 2009a).

6.7.3 *Geraniaceae*

The Geraniaceae family includes 7 genera and 841 species (http://www.theplantlist.org/) of annual and perennial herbs distributed all over the world and extensively cultivated for ornamental purposes. The plants show nodose stems and alternate or opposite, palmate, lobed leaves. Flowers are regular, pentamerous, lonely or grouped in inflorescences. The fruit, with a birdlike shape, is a schizocarp composed of five nutlets. The genus *Pelargonium* is of great importance for the production of EOs.

6.7.3.1 *Pelargonium roseum* Willd

P. roseum, also known as Reunion Geranium or Geranium-Rose, is an aromatic herb native to Southern Africa but occurring in other parts of the world where it is extensively cultivated owing to its pleasant rose-like scent. Indeed, its leaves are covered with capitate trichomes secreting an EO characterized by citronellol and geraniol giving a pleasant rose scent (Baser and Buchbauer 2015; Lis-Balchin et al. 1996). In the traditional medicine, the EOs of *Pelargonium* spp. are used as popular aromatherapy agents to treat stress, anxiety and depression (Setzer 2009; van der Watt et al. 2008; Lemon 2004; Dobetsberger and Buchbauer 2010). Several bioactivities including antimicrobial, antifungal, insecticidal and anti-inflammatory have been attributed to this EO (Carmen and Hancu 2014; Lis-Balchin 2003; Rezaei et al. 2008; Tabari et al. 2017c). Notably the *P. roseum* EO showed larvicidal and ovicidal effects on *Cx. pipiens* with LC_{50} of 5.5 and 0.5 ppm, respectively (Table 6.1) (Tabari et al. 2017b). Its main constituents, namely, citronellol and geraniol, were also toxic on larvae of *Ae. aegypti*, showing LC_{50} of 49.9 and 49.3 ppm, respectively (Table 6.2) (Ali et al. 2013).

6.7.4 *Lamiaceae*

The family of Lamiaceae (syn. Labiatae) includes herbs or shrubs endowed with quadrangular stems and simple leaves in opposite pairs. The flowers are bisexual and zygomorphic, forming inflorescences called verticillasters and spikes where florets are grouped in axillary or apical condensed and branched cymes, respectively. They are characterized by a two-lipped gamosepalous calyx and a tubular two-lipped corolla, four stamens and a four-lobed ovary giving rise to a schizocarp formed by four separated nutlets. The family encompasses 245 genera and 7886 species distributed in tropical and warmer temperate regions, mostly in Mediterranean and Asian countries (Allabi 2004). Lamiaceae species are used in industrial applications, e.g. as flavourings, antiseptics, digestive, expectorant and

preservative agents (Leung and Foster 1996). The Lamiaceae family is currently recognized as closely related to Verbenaceae and is divided into two major groupings, namely, Lamioideae and Nepetoideae, being specialized in the production of nonvolatile iridoids and monoterpene-containing EOs, respectively (Wink 2003). Actually, this family is the most cited in literature as a source of insecticidal compounds (Boulogne et al. 2012).

6.7.4.1 *Mentha* x *piperita* L.

M. x *piperita*, also called peppermint, is a hybrid generated by crossing *M. aquatica* L. with *M. spicata* L. It is a perennial herb with quadrangular, purple-red stems and flowers grouped in verticillasters arranged in terminal spikes (Abbaszadeh et al. 2009). *M.* x *piperita* is the most important mint species on a commercial level. Its EO is obtained from leaves and is characterized by menthol and menthone which are used to treat skin inflammations, respiratory problems, fever, muscle pains and in perfumery as flavour agents (Kumar et al. 2011). The peppermint EO is sold at different prices depending on the countries, e.g. 9 €/kg in China, 30–35 €/kg in India and 40–45 €/kg in the USA (Lubbe and Verpoorte 2011). Peppermint EO has shown larvicidal effects against *Ae. aegypti* and *Cx. pipiens*, with LC_{50} of 47.2 and 40.3 ppm, respectively (Amer and Mehlhorn 2006; Michaelakis et al. 2011), as well as repellent activity against *An. annularis*, *An. culicifacies* and *Cx. quinquefasciatus* (Table 6.1) (Ansari et al. 2000).

6.7.4.2 *Ocimum basilicum* L.

O. basilicum, also known as basil, is an aromatic herb native to Asia but widely cultivated in the Mediterranean area as a flavouring agent and source of EO. Regarding the latter, several chemotypes are reported, with those containing high levels of methyl chavicol and linalool as the most important ones (Varga et al. 2017). The EO is used to make perfumes and household cleaning products or as flavouring in foodstuffs (Putievsky and Galambosi 1999); its price has been estimated to be in the range 40–45 €/kg. Basil EO is recognized as antioxidant, anaesthetic, anti-inflammatory, antimicrobial and antiproliferative agent (Rodrigues et al. 2017; Varga et al. 2017). The basil EO showed a wide spectrum on larvicidal effects, being effective on *Cx. tritaeniorhynchus*, *Cx. quinquefasciatus, Ae. albopictus* and *An. subpictus* with LC_{50} of 14.0, 40.0, 12.0 and 9.8 ppm, respectively (Table 6.1) (Govindarajan et al. 2013; Rajamma et al. 2011). Furthermore, the basil EO exerted ovicidal and repellent effects on *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* (Phasomkusolsil and Soonwera 2012). The main basil EO constituents such as methyl chavicol and methyl eugenol were larvicidal on *Ae. aegypti* with LC_{50} of 46.4 and 36.5 ppm, respectively (Rocha et al. 2015; Tabanca et al. 2013).

6.7.5 *Lauraceae*

Lauraceae is one of the oldest families belonging to the angiosperm dicotyledons appearing approximately more than 100 million years ago. It includes 68 genera and 2978 species, mainly evergreen trees or shrubs growing in tropical and subtropical regions. Lauraceae species are strongly aromatic; they show simple, opposite, hard leaves bearing many glandular pockets containing EOs. Flowers are small and grouped in umbel-like inflorescences. The most important genera for EOs trade are *Cinnamomum* and *Laurus*.

6.7.5.1 *Cinnamomum verum* J. Presl

C. verum, also known as cinnamon, is an aromatic evergreen tree, 10–15 m tall, native to Sri Lanka and Western India and cultivated in tropical and subtropical regions. The bark of the tree, devoid of cork, is used to make the spice cinnamon as well as traditional Chinese remedy to treat fever, inflammations, cough and cardiovascular disorders (Wong et al. 2016). From the bark is also obtained the EO, whose price ranges from 155 to 230 €/kg (Lubbe and Verpoorte 2011), which is widely used on an industrial level, e.g. for preparation of pharmaceutics, seasonings, cosmetics, food and beverages (Li et al. 2013). The major component of this oil is the aromatic cinnamaldehyde which is responsible for important biological effects, namely, anti-inflammatory (Chao et al. 2005), antioxidant (Murcia et al. 2004) and antibacterial (Chang et al. 2001). The cinnamon EOs are obtained from bark and leaves and are characterized by cinnamaldehyde and eugenol, respectively. They exerted relevant larvicidal effects on *An. tessellatus*, *Cx. quinquefasciatus* and *Ae. aegypti*, with LC_{50} of 0.3, 0.7 and 2.3 and 1.0, 2.1 and 1.6 ppm, respectively (Samarasekera et al. 2005). Cinnamaldehyde exhibited larvicidal effects on *Ae. aegypti* and *Ae. albopictus*, with LC_{50} of 29.0 and 48.1 ppm, respectively (Table 6.2) (Cheng et al. 2004, 2009b). Eugenol was a noteworthy larvicidal against *Cx. pipiens* and *Ae. aegypti*, with LC_{50} of 18.3 and 33.0 ppm, respectively (Table 6.2) (Kimbaris et al. 2012; Cheng et al. 2004)

6.7.5.2 *Laurus nobilis* L.

L. nobilis, also known as Mediterranean bay, is a small aromatic tree growing wild in the Mediterranean area and widely cultivated elsewhere. Its leaves have a strong smell and bitter flavour; the lamina is sprinkled by many oil glands (idioblasts) storing the EO whose major constituent is 1,8-cineole. Traditionally, the leaves of Mediterranean bay are used to treat digestive and respiratory problems (Qnais et al. 2012). The EO, rich in 1,8-cineole and α-terpinyl acetate, is used on an industrial level to make perfumes and soaps (Kosar et al. 2005). This EO is endowed with antibacterial, antifungal and antioxidant activities (Bakkali et al. 2008). It also

showed larvicidal effects on *An. stephensi* and *Cx. pipiens* with LC_{50} of 14.9 and 16.5 ppm, respectively, (Mohammadreza 2010) and repellent effects against *Ae. aegypti* (Table 6.1) (Tabanca et al. 2013).

6.7.6 *Myrtaceae*

This family encompasses 145 genera and 5970 species distributed in warmer temperate, tropical and subtropical regions. It includes mostly evergreen shrubs or trees endowed with aromatic leaves due to the presence of schizolysigen pockets storing EOs and flowers usually clustered in inflorescences. The most important genera are *Syzygium*, *Eucalyptus*, *Melaleuca* and *Myrtus*.

6.7.6.1 *Syzygium aromaticum* (L.) Merr. & L.M. Perry

S. aromaticum, also known as clove, is an aromatic, evergreen tree native to Moluccas Islands and cultivated in Tanzania, Madagascar and Indonesia (Trease and Evans 1985). The peculiarity of this species is to produce flowers which are grouped in cymes at the end of twigs; they have a clove-like red calyx made up of four red and fleshy sepals, whereas petals are caducous. In the Ayurvedic medicine, clove has been used to treat toothache, digestive problems, skin diseases and as anaesthetic (Khan and Ahmad 2012). The dried flower buds are rich of EO with yields above 10% (w/w) and eugenol as the major constituent; this EO accounts for 5–6 €/kg (Lubbe and Verpoorte 2011). The clove EO is effective against oral bacteria associated with dental caries and periodontal disease (Cai and Wu 1996). The clove EO has been reported as larvicidal against *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} of 21.0 and 15.0 ppm, respectively (Table 6.1) (Costa et al. 2005). Furthermore, it exhibited repellent and ovicidal effects against *Cx. quinquefasciatus Ae. aegypti* and *An. dirus* (Suwansirisilp et al. 2013; Phasomkusolsil and Soonwera 2012).

6.7.6.2 *Eucalyptus* spp.

Known as eucalyptus, species of the *Eucalyptus* genus are giant trees native to Australia and distributed in tropical and subtropical regions (Filomeno et al. 2017). Some of them are cultivated all over the world owing to their quick growth and capacity of drying wet soils. They bear aromatic, coriaceous, opposite or alternate leaves endowed with schizogenic secretory pockets (Vuong et al. 2015). Main representatives of this genus are *E. globulus* Labill., *E. camaldulensis* Dehnh. and *E. citriodora* Hook. The leaves contain up to 3.5% (w/w) of EO whose price ranges from 6 to 35 €/kg depending upon species and geographic origin (Lubbe and Verpoorte 2011). Eucalyptus EOs, mainly those characterized by 1,8-cineole, are endowed with antiseptic, expectorant, mucolytic and nasal decongestant and are used in the manufacturing of syrup, tablets, nasal drops and inhaled preparations to be used in respiratory disorders (Hassine et al. 2012). Eucalyptus EOs have been recognized as GRAS by the US EPA since they have

high LD_{50} on rats (Batish et al. 2008). The EO of *E. camaldulensis*, rich in α-pinene, α-phellandrene and *p*-cymene, exhibited larvicidal effects on *Ae. aegypti* and *Ae. albopictus*, with LC_{50} of 31 and 55 ppm, respectively (Table 6.1) (Cheng et al. 2009a). The EO of *E. citriodora*, which is characterized by citronellal and citronellol, exerted ovicidal effects on *An. dirus* and *Cx. quinquefasciatus*, larvicidal effects on *An. gambiae* (LC_{50} of 33.7 ppm) and repellent effects on *An. arabiensis* (Table 6.1) (Phasomkusolsil and Soonwera 2012; Bossou et al. 2013; Solomon et al. 2012).

6.7.7 Poaceae

The Poaceae family is one of the most successful groups in the angiosperm, being widespread all over the world. It encompasses 759 genera and 9700 species of annual and perennial herbs able to grow in any environmental condition. The plants exhibit alternate, linear, sessile, parallel leaves with a sheath rounding the stem and a ligule at the junction of sheath and leaf blade. Inflorescences are very varied but are usually composed of units (spikelets) with a pair of sterile glumes at the base of each spikelet. Fruit is a one-seeded caryopsis, mostly edible (cereal) due to the high content of starch and proteins. From an economic perspective, it is the most important family to humans as a source of cereals containing nutrients like starch and proteins. The most important genera for the production of EOs are *Cymbopogon* and *Vetiveria*.

6.7.7.1 *Cymbopogon citratus* (DC.) Stapf

C. citratus, also known as lemongrass, is a perennial aromatic herb native to Southeastern Asia and widely cultivated in tropical regions. Leaves are rich of EO which is characterized by geranial, neral and myrcene. The EO is used in cosmetics and perfumes; its price is estimated in the range 10–12 €/kg. The EO exerted a wide spectrum of effects, namely, larvicidal against *An. funestus* and *An. gambiae* (Ntonga et al. 2014; Bossou et al. 2013), repellent against *Cx. quinquefasciatus* (Suwansirisilp et al. 2013) and ovicidal against *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* (Table 6.1) (Phasomkusolsil and Soonwera 2012).

6.7.8 Rutaceae

The family of Rutaceae includes 158 genera and 1730 species of shrubs and trees distributed in temperate and tropical regions. They show simple, opposite leaves, endowed with schizolysigen secretory pockets containing EOs. The latter occur also in green stems and in the fruit epicarp (peel), also known as flavedo. Flowers are usually grouped in cymex. Fruits are of various natures, being those of the *Citrus* genus known as hesperidia. *Citrus* is the most important genus for the production of EOs that are obtained by cold pressing from the fruit peel.

6.7.8.1 *Citrus* x *aurantium* L.

C. x *aurantium*, also known as bitter orange, is a small tree, 4–5 m tall, native to North India and widely cultivated also in temperate regions. The plant is endowed with thorny twigs and coriaceous, shiny, oval leaves containing schizogenic pockets. Flowers are white, scented, and grouped at the axilla of leaves. The fruit, which is smaller than that of sweet orange, has a wrinkled, orange-red epicarp (peel), containing many schizogenic pockets secreting EO. The latter is rich in limonene, myrcene and α-pinene; the price of EO is around 45–50 €/kg. Flowers are a source of EO known as neroli oil, containing linalool, linalyl acetate, limonene and β–pinene; neroli oil is used in perfumery and pharmaceutical preparations (Leung and Foster 1996). The EO obtained from the peel and aerial parts is dominated by limonene and is highly effective against larvae of *An. stephensi*, *An. labranchiae* and *Cx. pipiens*, with LC_{50} of 31.2, 22.6 and 39.8–52.0 ppm, respectively (Table 6.1) (Sanei-Dehkordi et al. 2016; Michaelakis et al. 2009; El-Akhal et al. 2015). Limonene is larvicidal on a wide spectrum of mosquitoes such as *An. stephensi*, *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*, with LC_{50} of 8.8, 12.0, 32.7 and 14.1, respectively (Table 6.2) (Govindarajan et al. 2012; Cheng et al. 2009a).

6.7.9 Verbenaceae

The family of Verbenaceae encompasses 34 genera and 2647 species distributed in tropical and subtropical regions and, to a minor extent, in temperate areas. It includes mostly annual or perennial herbs with simple, opposite or whorled leaves. Flowers, endowed with two-lipped corollas, are grouped in inflorescences such as racemes and spikes. The most important genera for the production of EOs are *Aloysia* and *Lippia*.

6.7.9.1 *Lippia* spp.

The *Lippia* genus includes shrubs native to South America and also widely cultivated in temperate regions for ornamental purposes. *Lippia* species are very important in the traditional medicine of Latin America where they are used to treat digestive problems, as sedative and antispasmodic agents. They are characterized by aromatic leaves which are endowed with many glandular trichomes secreting EOs. The most important species of the genus are *L. alba* (Mill.) N.E.Br. ex Britton & P. Wilson and *L. multiflora* Moldenke. *L. multiflora* is native to central and occidental Africa where it is used in the traditional medicine to treat inflammatory diseases (Soro et al. 2016). The EO is obtained from the leaves and is characterized by several chemotypes, namely, thymol, citral, 1,8-cineole, linalool, α-terpineol and nerolidol (Soro et al. 2016). Several biological activities are recognized for this EO, namely, sedative, anti-infective, antitussive, antispasmodic and hypotensive (Soro

et al. 2016). In addition, the *L. multiflora* EO has been used as a coadjuvant in the treatment of malaria (Valentin et al. 1995). The EO of *L. multiflora,* notably the thymol chemotype, was proven to be ovicidal against *An. gambiae* with LC_{50} of 17.1 ppm (Table 6.1) (Bassolé et al. 2003).

6.7.10 Zingiberaceae

The Zingiberaceae family includes aromatic perennial herbs endowed with stout rhizomes and regular flowers grouped in branched inflorescences. It encompasses 52 genera and 1587 species distributed in tropical regions and equatorial forests of Southeastern Asia. The main genera of commercial interest are *Alpinia*, *Kaempferia*, *Zingiber*, *Elettaria* and *Curcuma*. They produce EOs mainly into the rhizomes.

6.7.10.1 *Zingiber officinale* Roscoe

Z. officinale, also known as ginger, is a perennial, aromatic herb native to India and extensively cultivated in China, Southeastern Asia and tropical Africa. Since ages ginger is used in the traditional medicine of China and India in the treatment of digestive problems, sickness and as an antiemetic agent (Yeh et al. 2014). The plant is endowed with a typed rhizome, lanceolate leaves and green, purple lipped flowers grouped in spikes. The aromatic, spicy rhizome contains EO which shows a chemical composition variable depending on the geographic origin. The most abundant components are α-zingiberene, *ar*-curcumene, β-sesquiphellandrene and citral (Racoti et al. 2017). Its price ranges from 70 €/kg in India to 130–140 €/kg in Sri Lanka (Lubbe and Verpoorte 2011). The ginger EO was proven as an ovicidal agent against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, with LC_{95} of 32.2, 52.7 and 53.4 ppm, respectively (Table 6.1) (Prajapati et al. 2005).

6.8 Strengths and Weakness

EOs are natural mixtures of volatile compounds occurring in aromatic plants with yields in the range of 0.5–2.0% so that they represent an ‘enriched extract’ which generally is more effective than many other kinds of extract on insects (Isman 2017). Many EOs are cheap and readily available due to their uses in other fields such as aromatherapy, food and beverages, fragrances, skincare and household cleaning products. Manufacturers dealing with EOs have the advantage to monitor their quali-quantitative composition by the use of up-to-date gas chromatography-mass spectrometry methodologies whose costs have significantly decreased in the last years (Isman 2017).

Being complex mixtures of bioactive compounds, EOs enjoy several advantages such as the wide spectrum of efficacy against mosquito vectors and other pests of medical and agricultural importance, the multiple mode of actions (e.g. different molecular targets in insects are addressed), the unlikely insurgence of resistance in insects and the low impact on the human and animal health and the environment. In fact, the rapid volatilization of EOs in the air reduces the risk to the environment compared with current synthetic insecticides. Furthermore, other predator and pollinator insect populations will be less affected by EOs as a result of their low residual activity.

On the other hand, these advantages are counterbalanced by several drawbacks that are currently limiting their spread and marketing. These weaknesses are linked to the nature of EOs. Indeed, the volatility of EO constituents limits their persistence in the environment so that requiring frequent reapplication when used out-of-doors. The lipophilicity of the molecules limits their applicability in wet environments where mosquitoes are usually breeding. Many EO constituents are highly instable when exposed to light air and high temperatures so that they give raise to degradation products devoid of efficacy. Being very sensitive to genetic and environmental factors, EOs may exhibit a significant intraspecific variability giving raise to several 'chemotypes' which can influence the whole biological efficacy. Moreover, as EOs get old, their overall quality tends to decrease as a consequence of alteration of their organoleptic attributes (e.g. odour, flavour, colour and consistency). Being comparable with 'highly concentrated extracts', EOs cannot be used as pure but only after carefully dilution in a proper solvent carrier. Indeed, EOs often contain several chemical compounds which are important contact sensitizers to skin such as linalool, limonene, benzyl benzoate, citral, geraniol, isoeugenol and eugenol (Uter et al. 2010).

All the above disadvantages highlight the need for efficient stabilization processes. The latter rely on the so-called encapsulation process through the development of an appropriate formulation. This process assures an improvement in the usability, efficacy and shelf life of EOs and their active compounds. Several formulation techniques have been developed in the last years to overcome the instability of EOs. They can be divided into three groups: (1) chemical processes or interfacial polymerization (Chung et al. 2013); (2) physico-chemical processes such as interfacial coacervation, oil-emulsion entrapment, β-cyclodextrin inclusion complexes and liposome encapsulation (Galvão et al. 2015; Dong et al. 2011); and (3) physical processes such as spray drying, co-crystallization, extrusion or fluidized bed coating (Fang and Bhandari 2010; Laohasongkram et al. 2011). Encapsulation methodologies are generally used to prepare micro- and nano-emulsions with a controlled release of EOs and their active ingredients (Sakulku et al. 2009; Cespi et al. 2017; Lopez et al. 2012; Sugumar et al. 2014; Vishwakarma et al. 2016). In addition, new technologies capable to mimic the natural defence strategies used by plants against insects, in terms of release and behaviour of the individual EO components, will be very useful in the next years (Miresmailli et al. 2013).

6.9 Concluding Remarks

In the last years, the basic research on botanical insecticides have focused on the discovery of new natural products (e.g. plant extracts and/or EOs) with toxic or repelling properties against mosquitoes. As a consequence, a large database including effective plant extracts, EOs and pure compounds has been created. On the other hand, limited research has been carried out on the optimization of extractive methods of plant extracts and EOs to be adopted on an industrial scale as well as on the development and field application of essential oil-containing formulations. Therefore, so far research has moved towards the discovery end leaving a gap concerning the industrial production and application of botanical insecticides. The latter aspects will constitute a fascinating challenge to pursue in the next years in order to fulfil the technology transfer end.

The industrial production of EO-containing botanical insecticides will be possible if the following requirements are fulfilled: (1) availability (generally in the order of tonnes per month) and sustainability of the plant source throughout the cultivation of insecticidal plants or, alternatively, by harvesting fast-growing weedy annual plants; (2) steadiness of the chemical profile (fingerprint) of EOs; (3) low costs for EOs so obtained, generally below 100 €/kg; (4) low costs for formulation development; (5) standardization of EO compositions; (6) low toxicity on nontarget organisms; (7) low impact on environment and human health; and viii) regulatory registration and approval.

As regarding the first aspect, insecticides derived from EOs obtained from rare species would not be easily produced due to shortage of plant material. Thus, priority should be given to EOs from readily available and easily cultivable species (McChesney 1994). In this respect, new opportunities could be offered to associations of small-scale farmers in developing countries to cultivate insecticidal plants based on a contractual basis as in the case of the production of pyrethrum in Eastern African countries (Isman 2017). Furthermore, the purchase of a small-scale extraction equipment is relatively cheap (e.g. a hydrodistillation system with a volume of 65L can be acquired with just under 2000 $). Relative to the chemical standardization of EO-containing products, the manufacturers should make homogeneous mixtures with a known level of bioactive compounds or create EO blends composed of bioactive pure constituents in order to obtain an effective combination. In addition, understanding the synergism between different EO constituents will allow to manufacture cheaper mixtures prepared using commercially available substances (Hummelbrunner and Isman 2001; Pavela 2008, 2014b). As regarding the regulatory aspect, this is still a barrier limiting the commercialization and use of EO-based insecticides. In this respect, an exemption list, including those EOs with historical uses in food and traditional medicine (like in the US), should be created to bypass this barrier in the EU.

In the near future, the costs for governmental registration of botanical insecticides should be lowered and the authorization process simplified (e.g. for EOs with documented use in the food and cosmetic industry) in order to assure their fast

spread and utilization by agrochemical companies. In addition, a tight collaboration between researchers and manufactures aimed at putting research findings into practice is needed.

The awareness of the efficacy of EO-based insecticides and of their use as alternative tools able to improve not only human health but also quality and safety of foods in growers and manufacturers will enable the market of natural insecticides to grow up in the next years.

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Chapter 7
Mouthparts of Bloodsuckers and Their Ability to Transmit Agents of Diseases

Heinz Mehlhorn

Abstract Many arthropods (mites, ticks, insects) that parasitize at the surface of vertebrates of humans by sucking blood or lymph often get into contact with inhabiting agents of disease that may lead to severe diseases or even to death of their hosts, if transmitted during the next bloodsucking act. These various agents of disease (e.g., prions, viruses, bacteria, protozoans, or worms) may be transmitted by simple transition by the help of contaminated mouthparts or after a permanently running reproduction inside the saliva or intestinal fluids of the aggressors. The present chapter lists different pathways and modes of transmission reaching from simple contacts to body fluids to development and use of sophisticated mouthparts that may besides their food uptake functions inject peculiar agents of diseases at definite sites (e.g., directly into blood vessels, into dermal tissues, etc.).

Keywords Transmission · Agents of disease · Adaptations of mouthparts · Viruses · Parasites · Bacteria · Fungi · Diseases

7.1 Introduction

Agents of disease of humans and animals belong to several groups of individuals: prions, viruses, bacteria, fungi, and parasites. The latter are organized as protozoans, helminths, or arthropods. While some protozoans and helminths have developed direct pathways of propagation from one host to another (e.g., oral uptake of fecally excreted tiny protozoan cysts or worm eggs), others need the help of bloodsucking arthropods (ticks, insects) or of bloodsucking worms (leeches). The present chapter summarizes several examples of already existing transmission cycles, while other pathways are either under development or are already existing but not yet sufficiently investigated.

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7.2 Mechanical Transmission

Adult flies (e.g., species of the genera *Musca*, *Sarcophaga*, *Lucilia*; Figs. 7.1, 7.2 and 7.3) often feed at the feces of humans and animals. Thereby they may ingest contained bacteria, cysts of protozoans, and worm eggs, which they may distribute onto the surface of animals or onto the food of animals and humans. The enormous potential of this transmission is shown in Fig. 7.4a, b, where a single *Musca* fly had been exposed to an agar surface. The short contact of 1 min led to an enormous transmission and growth of bacteria. The bacteria had apparently become attached at the feet and at the stamp-like labella of the flies (Figs. 7.5, 7.6 and 7.7). When exposing clean flies to food containing artificially added nematode eggs or oocysts of coccidians, it was later possible to isolate unchanged specimens of these parasitic stages from their feces. These experiments prove that flies are able to transport and distribute mechanically besides bacteria and viruses also agents of diseases and thus to induce diseases. Thus flies have to be kept away from human dwellings and stables.

Fig. 7.1 Macrophoto of an adult fly (*Sarcophaga carnaria*)

Fig. 7.2 Macrophoto of an adult fly (*Musca domestica*)

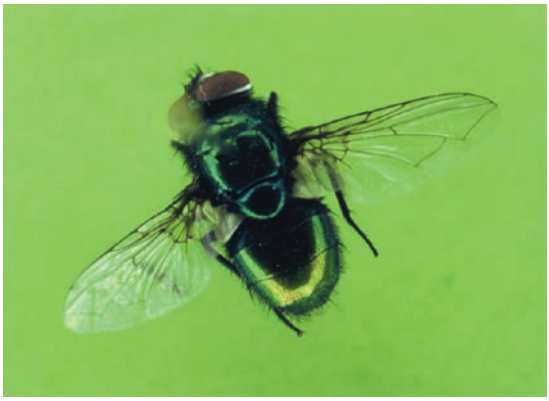

Fig. 7.3 Macrophoto of an adult flesh fly (*Lucilia sericata*)

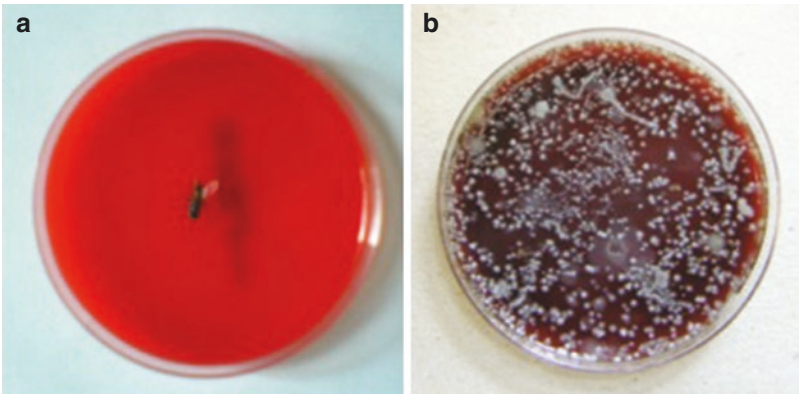

Fig. 7.4 Macrophotos of an agar culture before (**a**) and some days after (**b**) exposition to a single fly for 1 minute showing the enormous growth of bacteria during the following 4 days

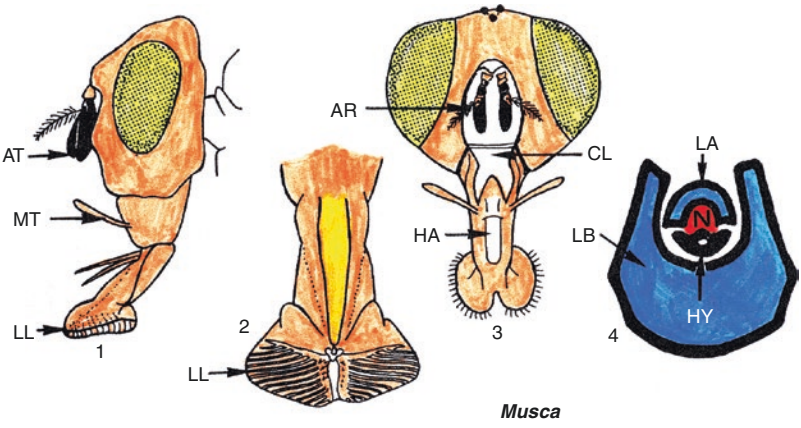

Fig. 7.5 Diagrammatic representation of the mouthparts of a licking-sucking fly (e.g., *Musca*). (**1**) Lateral aspect of the head. (**2**) Ventral aspect of the labellum. (**3**) Frontal aspect of the head (note the three ocelli at the top of the head). (**4**) Cross section through the labellum. *AR* arista; *CL* clypeus; *HA* haustellum; *HY* hypopharynx with saliva channel; *LA* labrum; *LB* labium; *N* food channel

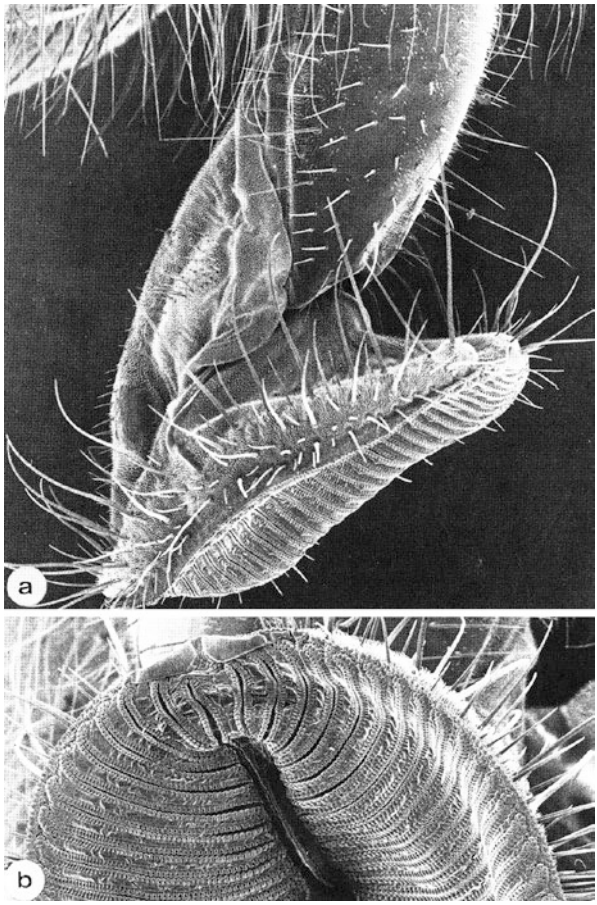

Fig. 7.6 Scanning electron micrographs of the dorsal (**a**) and ventral (**b**) aspect of the labellum of the mouthparts of a *Calliphora* fly, which excrete glueing substances and ingest them with the food

A similar process of mechanical transmission of agents of diseases may occur during the bloodsucking process of leeches. This potential was demonstrated by experiments of our group (Nehili et al. 1994), when uninfected leeches of the species *Hirudo medicinalis* were allowed to suck blood at mice, which had been previously infected by stages of *Trypanosoma brucei* or *Toxoplasma gondii* (Fig. 7.8). It turned out that these leeches had been able to transmit these ingested agents of disease (even after months of starving) to parasite-free laboratory mice. Furthermore examinations of leeches obtained from rivers in Africa showed in the same series of studies that they contained blood being positive for AIDS viruses. These findings indicate that potential mechanical transmissions should be taken more intensely into consideration, if patients show unclear signs of disease.

Another example for potential mechanical transmissions may be seen when considering the painful feeding process of biting and bloodsucking flies such as *Stomoxys calcitrans* (Figs. 7.9 and 7.10). In contrast to most other bloodsucking

Fig. 7.7 Macrophoto of the ventral side of flies showing their feet which may become contaminated when sitting on feces

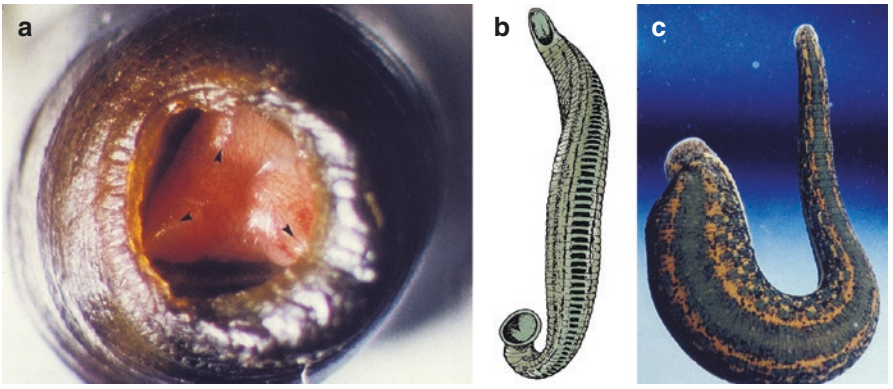

Fig. 7.8 (**a**) Macrophoto of the anterior sucker showing three rows of fine teeth. (**b**) Diagrammatic representation of the *Hirudo* leech. (**c**) Macrophoto of a *Hirudo* leech from dorsal

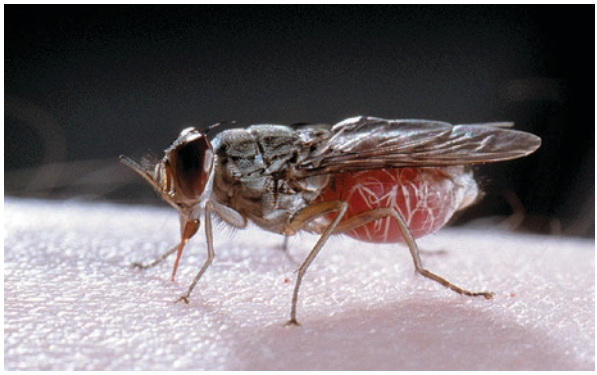

Fig. 7.9 Macrophoto of the biting fly *Stomoxys calcitrans*. The bloodsucking apparatus is injected into the skin

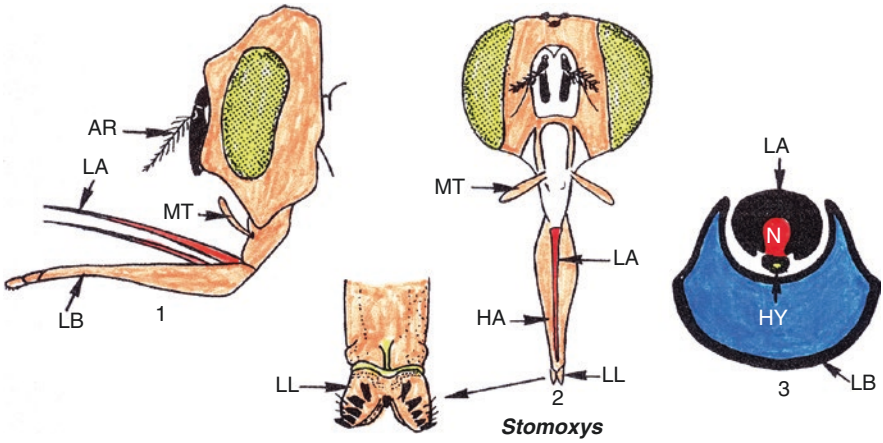

Fig. 7.10 Diagrammatic representation of the head and the bloodsucking systems of the bloodsucking fly *Stomoxys calcitrans*. (**1**) Lateral aspect of the head. (**2**) Frontal aspect of the head and the sucking system. (**3**) Cross section through the bloodsucking system. *AR* arista; *HA* haustellum; *HY* hypopharynx containing the saliva; *LA* labrum; *LB* labium; *LL* labellum; *MT* maxillary sensilla; *N* food channel

insect species, where exclusively females ingest blood, both sexes of *Stomoxys calcitrans* and some related species engorge blood of their hosts. Although there are rather few definitively proven cases, where *Stomoxys* stages have transmitted agents of diseases such as those of poliomyelitis, sleeping sickness, or various bacteriosis, the chances of transmissions are rather high, since these bloodsuckers are able to proceed a quick host change when being disturbed during their blood meal. In such cases agents of disease being attached (inside host blood) at their mouthparts would be injected into the new host. Such processes could become especially common in camps of soldiers, refugees, and other groups of persons staying close together outside of houses.

Similar pathways of transmission of agents of diseases may also become initiated, when huge numbers of bedbugs (*Cimex lectularius*, family Cimicidae) occur

in overcrowded refugee camps and thus get the chance to wander from bed to bed and inject their rather large sucking mouthparts into the skin of different sleeping persons (Figs. 7.11a–c and 7.12). If the mouthparts are covered by remaining agents of disease from a previous sucking act, they can become easily mechanically transmitted.

In contrast to bedbugs, all stages of the so-called raptor bugs (Reduviidae, Fig. 7.13) are able to transmit cyclically agents of disease such as the flagellate *Trypanosoma cruzi* (Fig. 7.14). The transmission, however, does not occur by injection of the infectious stages within the saliva but by excreting them with their feces. Then the hosts may rub them into the biting channels of the itching skin regions. This pathway of transmission is still today very successful in South and Central America, where such large bugs are common in houses of the poor rural population. The WHO estimated still in 2017 about 40 million cases of the human Chagas disease.

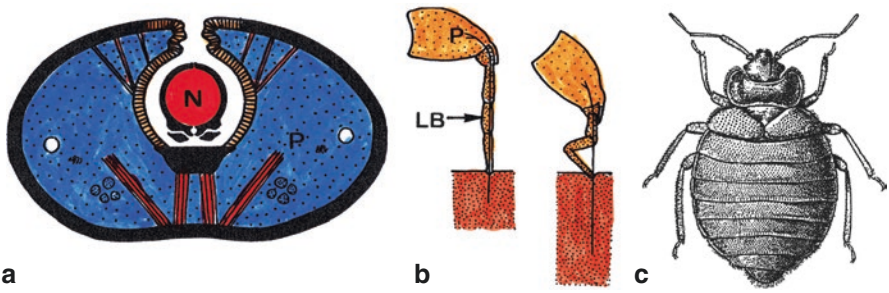

Fig. 7.11 (**a**–**c**) Diagrammatic representation of the mouthparts and the dorsal side of the bedbug *Cimex lectularius*. (**a**) Cross section through the proboscis covering inside the mouthparts. (**b**) Phases of entering the sucking structures (maxillae, mandibles). (**c**) Dorsal side of a female bug. The remnants of the wings are seen at the dorsal side of the body. *AN* anus; *AT* antennae; *AU* eye; *B* Berlese organ; *C* coxae of legs; *CX* coxa of anterior leg; *KL* claw; *LB* labium; *M* central breast; *MS* metasternum; *N* food channel; *P* proboscis; *PS* prosternum; *RU* remnant of the wings seen from below; *STI* stigma; *TA* tarsus; *TI* tibia

Fig. 7.12 Light micrograph of a bedbug excreting two eggs

Fig. 7.13 Macrophoto of a raptor bug of the genus *Triatoma* sp. (vector of, e.g., *Trypanosoma cruzi*)

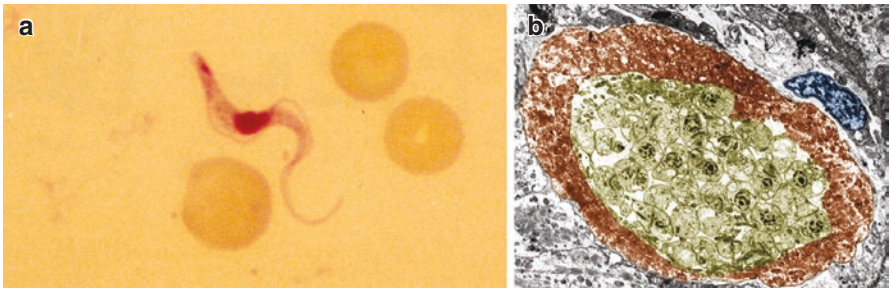

Fig. 7.14 (**a**) Light micrograph of a Giemsa-stained *Trypanosoma cruzi* stage in human blood. (**b**) Electron micrograph of a section through a heart muscle cell containing *T. cruzi* stages

7.3 Cyclic Transmission of Agents of Disease

It seems reasonable that agents of disease that occur in blood and/or lymph fluid of humans and animals had originally been transmitted by other modalities than during bites of ticks or insects, since a host change needs a considerable adaptation to physiological conditions in a new (second) host. Nevertheless there are actually large numbers of important well-known agents of disease that are apparently since long commonly transmitted between several quite different hosts (e.g., man-ticks, man-mosquitoes). Probably many other cyclic transmissions are under development and will find their hosts in the next future especially in the present times of ongoing climate change and proceeding human migration.

7.3.1 Ticks and Mites

The mouthparts of bloodsucking ticks and mites are rather simple and uniform in the different species when compared to those of the different groups of insects (Figs. 7.15 and 7.16), since they consist of a hook-bearing hypostome, which is injected into the skin of a host and fixed therein by hooks. Then the two knife-like cheliceres start sawing movements thus cutting the walls of blood vessels leading to a small "blood lake," which is kept fluid by excretions of the salivary glands. These excretions may be

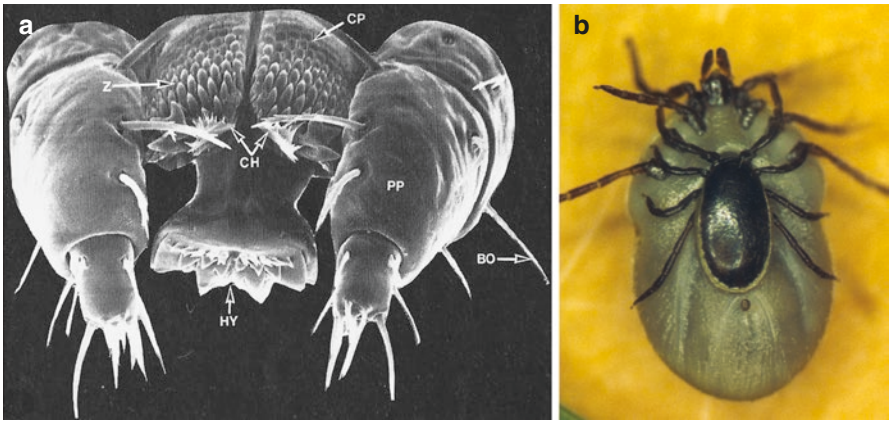

Fig. 7.15 (**a**) Scanning electron micrograph looking at the mouthparts of a tick of the species *Amblyomma variegatum* showing the so-called capitulum. *BO* sensitive bristles; *CH* cheliceres; *CP* tergum of the capitulum; *HY* hypostome; *PP* pedipalps; *Z* teeth-like scales on the sheath of the cheliceres. (**b**) Macrophoto of a fully sucked female and a male of the tick species *Ixodes ricinus* in copulation

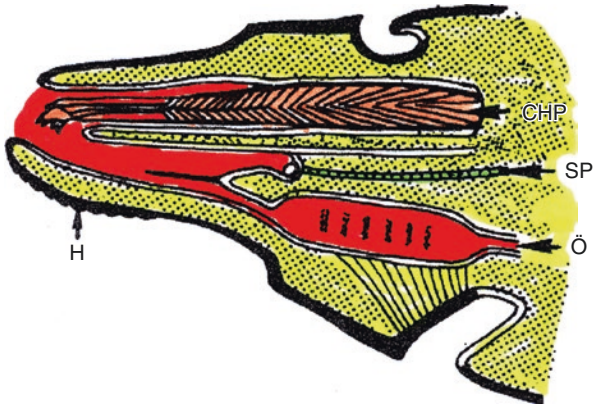

Fig. 7.16 Diagrammatic representation of a longitudinal section of the anterior end of a tick showing the arrangement of the mouthparts. *CHS* sheath of the cheliceres, which are protrusible and produce hollows in the host skin by back-and-forth movements; *SP* saliva ductule; *Ö* esophagus with muscles that initiate sucking movements

contaminated by agents of disease such as viruses (e.g., those of the early spring encephalitis) or bacteria (e.g., *Borrelia burgdorferi*). Table 7.1 lists some very important agents of disease, which can be transmitted by argasid or ixodid ticks worldwide.

The mouthparts of *mites* are similar to those of *ticks* and also rather simple when compared to the sophisticated ones of the various insect species. Figure 7.17 shows the rather similar mouthparts of various common mites attacking humans and animals. Most of them cut off small pieces from the skin of their hosts. Only a few of the mite species have transformed their mouthparts to become able to suck blood (e.g., *Dermanyssus* species) or lymph (*Neotrombicula autumnalis*, Fig. 7.17b). Again others enter the skin (epidermis) of their hosts and induce, e.g., scabies (Table 7.2).

7.3.2 Insects

7.3.2.1 Lice

Humans and animals are attacked by the developmental stages of several lice species, which all are wingless and suck blood in all developmental stages (adults, larvae, nymphs). The absence of wings is apparently an adaptation to their feeding behavior hidden in the hair of their hosts. With respect to human lice, there exist two groups:

1. **Body lice** (*Pediculus humanus corporis*, Fig. 7.18), which are able to stay several (up to 10) days away from the warm body of humans reaching a size of up to 5 mm. Due to this ability, they are adapted at common host changes, which enable them to transfer agents of disease from one host to another. Thus bacteria like *Rickettsia prowazekii* (agents of the louse-borne spotted fever) can easily be transmitted by all stages (larvae, nymphs, adults). When ingested during blood-sucking, these bacteria may reproduce themselves inside intestinal cells. Then they are excreted within the feces of lice and finally potentially inhaled by humans when shaking, e.g., bed covers.

Table 7.1 Examples of important ticks and potentially transmitted agents of diseases while sucking blood at the skin of their hosts

Ticks Family/species	Length (mm) of unfed adults (♀ fed)	Hosts in life cycle	Common host	Transmission of agents of diseases	Agent of disease
Argasidae					
Ornithodoros moubata	♀ 10 (18) ♂ 8	Many, not in cycles	Many, *humans*	*Borrelia duttoni* (tick relapsing fever)	S
Argas persicus *Argas reflexus*	♀ 5.5 (11) ♂ 5.5	Many, not in cycles	Birds, *humans*	*Borrelia anserina*	S
Ixodidae					
Ixodes ricinus	♀ 2.8–3.4 (8) ♂ 2.8–4	3	*Humans*, cats, dogs	Tick encephalitis, *Babesia* species, *Borrelia* species	V P S
Dermacentor marginatus *D. reticulatus*	♀ 5 (16) ♂ 5	3	*Humans*, cattle, sheep, dogs	Tularemia, Rickettsiosis, Anaplasmosis, Dog babesiosis	B R A P
Boophilus annulatus	♀ 2.5 (8)	1	Cattle	Texas fever (*Babesia bigemina*)	P
Amblyomma variegatum	♀ 6–7 (20) ♂ 5–6	3	*Humans*, cattle	Tularemia, Rocky Mountain spotted fever, Theileriosis	B R P
Hyalomma anatolicum	♀ 4–6 (14) ♂ 4–6	2–3	Cattle	Theileriosis	P
Rhipicephalus sanguineus	♀ 2–3 (6–7)	3	Dogs, *humans*	Rickettsiosis, Babesiosis	R P
Rhipicephalus appendiculatus	♀ 3–4 (10)	3	Cattle	East Coast fever: *Theileria parva*	P
Haemaphysalis punctata	♀ 3–4 (7)	3	Cattle, sheep, *humans*	Meningoencephalitis, piroplasmosis	V P

A Anaplasma; *B* bacteria; *P* protozoans; *R* rickettsiales; *S* spirochaetes; V viruses

2. **Head lice** (*Pediculus humanus capitis*) (2–3.5 mm; Fig. 7.19a, b) and so-called **pubic lice** (*Phthirus pubis*) (1–1.7 mm; Fig. 7.20). Both species are fixed at preferred body regions of humans. While *P. h. capitis* is mainly found inside the hair of their hosts, *P. pubis* occurs mainly on hair along the primary sexual organs of humans only rarely elsewhere (e.g. on the eyelashes). Due to the fact that mainly single fertilized females switch from one host to another, transmissions of agents of disease are rather rare and only documented in a few cases. In principle it occurs only—if at all –, when bloody mouthparts are immediately injected into the skin after a quick host change. These mouthparts are hidden inside a snout-like formation of the labrum, which contains the labium, the maxillae and the

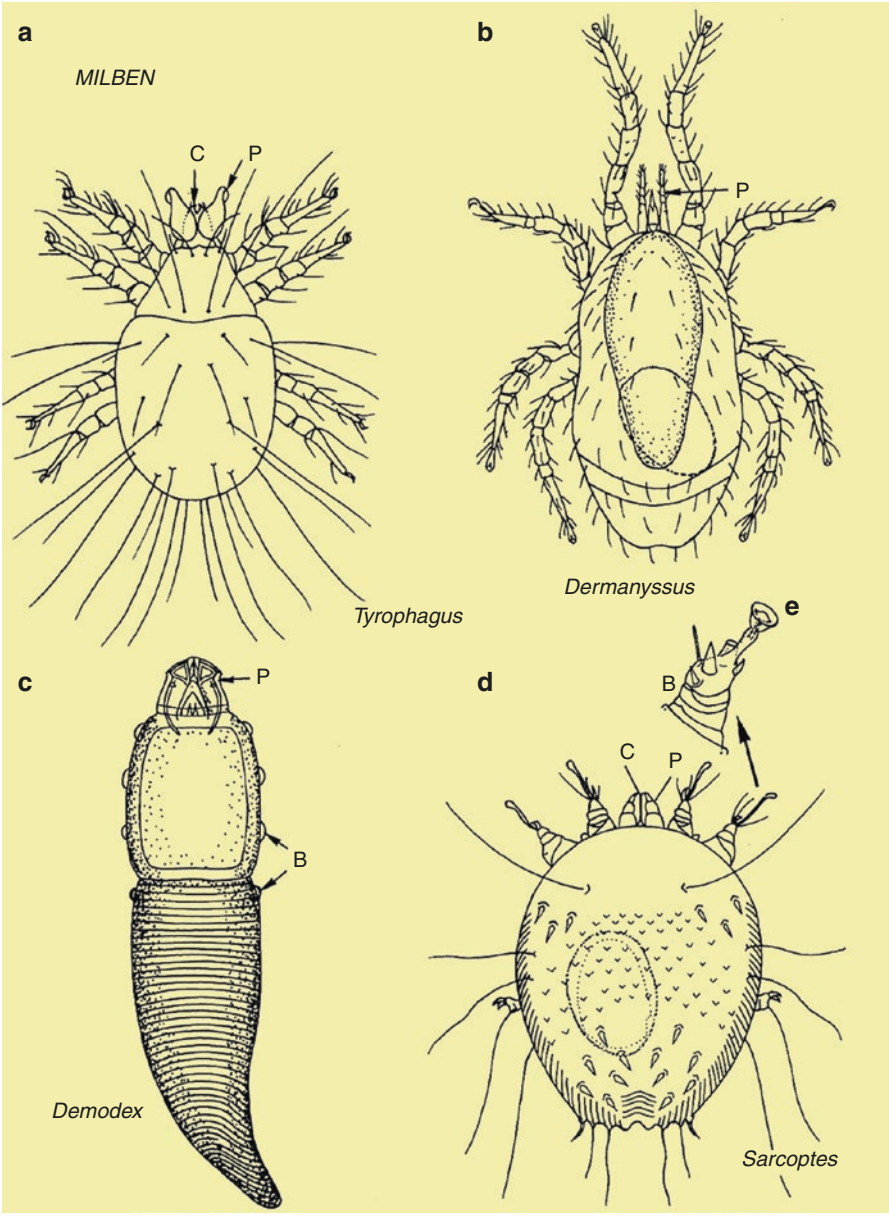

Fig. 7.17 Diagrammatic representation of common and (for humans) important mite species (seen from their dorsal side) (**a**) *Tyrophagus putrescentiae* male (lives, e.g., in flour). (**b**) *Dermanyssus gallinae* (♀) containing an egg. These mites suck blood at humans and animals. (**c**) *Demodex phylloides*. These mites stick inside the bases of hair of vertebrates. (**d**) *Sarcoptes scabiei*. These mites dig channels in the epidermis of vertebrates including humans. (**e**) Leg of the cat dermal mite *Notoedres cati*, which induces notoedric mange. *B* short leg; *C* cheliceres; *P* pedipalps

Table 7.2 Some common species of mites endangering humans and/or animals

Species	Size (adults) (mm)	Hosts	Vectorship or introduced disease
Glycyphagus domesticus	♀ 0.4–0.8 ♂ 0.3–0.5	*Humans*[a]	Allergy due to skin contact (so-called grocer's itch)
Acarus (Tyroglyphus) siro	♀ 0.4–0.6 ♂ 0.4	*Humans*[a]	Baker's itch
Dermatophagoides pteronyssinus	♀ 0.4 ♂ 0.4	*Humans*[a]	Dermatosis, allergic asthma
Dermanyssus gallinae	♀0.7 ♂0.6	Chicken, birds, *humans*	Chicken anemia; St. Louis encephalitis of humans (*V*)
Trombicula akamushi (Asia)	Larva 0.2–0.5	*Humans*	Tsutsugamushi fever (*R*)
Neotrombicula autumnalis (Europe)	Larva 0.2–0.5	*Humans*, many animals	Spring-autumn dermatosis (allergic reaction)
Sarcoptes scabiei	♀ 0.3–0.5 ♂ 0.2–0.3	*Humans*, animals	Scabies
Demodex folliculorum	♀ 0.4 ♂ 0.3	*Humans*	Rosacea
Notoedres cati	♀ 0.2–0.3 ♂ 0.1–0.15	Cats	Notoedric mange
Otodectes cynotis	♀ 0.4–0.5 ♂ 0.3–0.4	Dogs	Otodectic otitis
Psoroptes species	♀ 0.6–0.8 ♂ 0.5–0.6	Ruminants	Psoroptic mange
Chorioptes species	♀ 0.4–0.6 ♂ 0.3–0.4	Ruminants	Chorioptic mange
Varroa jacobsoni	♀ 1.2–1.7 ♂ 0.8	Bees	Larval death, bee flu

R rickettsiosis; *V* viral disease
[a]This disease is a result of an allergic reaction

Fig. 7.18 Scanning electron micrograph of a body louse (*Pediculus humanus corporis*) and three eggs

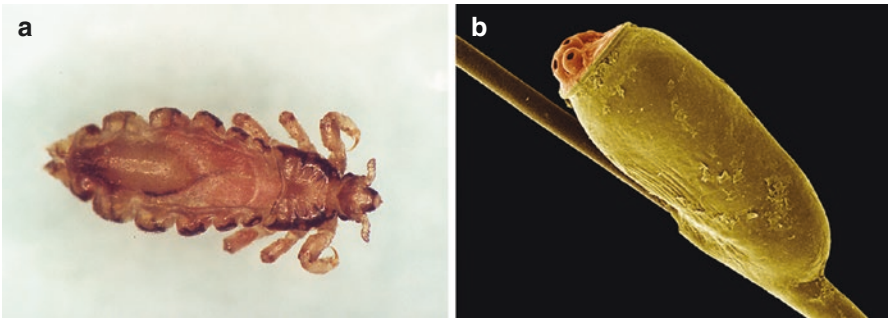

Fig. 7.19 (**a**) Macrophoto of a female head louse (*Pediculus humanus capitis*) containing an egg. (**b**) Scanning electron micrograph of an egg of *P. h. capitis* attached at hair

Fig. 7.20 Macrophoto of a pubic louse (*Pthirus pubis*)

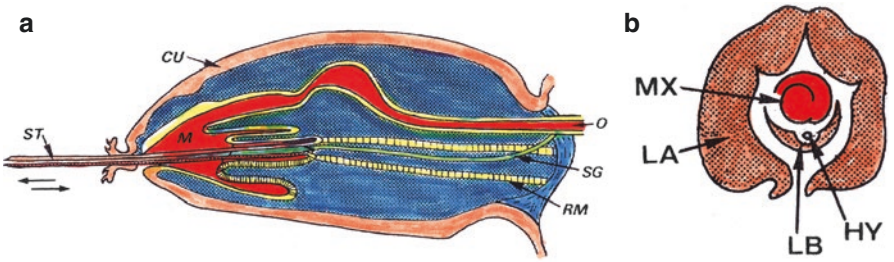

Fig. 7.21 Diagrammatic representation of the head of lice (**a**) showing the protrusible sucking stilet (comprising labium and maxillae) and (**b**) the cross section of the snout. *CU* cuticle; *HY* hypopharynx; *LA* labrum; *LB* labium; *MH* mouth hollow; *MX* maxillae; *O* esophagus; *RM* retractor muscle; *SG* saliva channel; *STL* retractible stilet (labium, maxillae)

hypopharynx, which engorges the sucked blood (Fig. 7.21a, b). The labium and the maxillae form together a channel (Fig. 7.21a, b), which is protruded into the skin at the beginning of the sucking and redrawn afterwards, so that the mouth appears again as a snout.

7.3.2.2 Fleas (Siphonaptera)

Fleas (Table 7.3) are laterally flattened, wingless, yellowish-brownish appearing insects, which possess a pair of extremely strong hind legs, which allow jumps of up to 30 cm in length in a speed of up to 2 Mach (Figs. 7.22, 7.23, 7.24 and 7.25). The different species reach a size of up to 7 mm in length. Both males and females suck blood, while larvae feed on organic particles on the soil. While male and female adults of most species are able to change frequently the host, the females of the so-called sand fleas (genus *Tunga*) enter the skin of humans and animals (Figs. 7.26 and 7.27), reach therein diameters of up to 1 cm, and thus may destroy toes (especially of children) and induce bacterial infections often leading to a loss of toes.

Fleas may host many parasitic or bacterial agents of diseases that are commonly transmitted either during injection of saliva or via contact to excreted feces. Well known are the transmissions of the bacterial agents of plague (*Yersinia pestis*) that have killed millions of humans in former centuries prior to the invention of the antibiotics (Kitasato 1894; Yersin 1894; Fleming 1929). The adult fleas suck blood of their hosts (94% at mammals, 6% at birds) by the help of their sophisticated mouthparts including a food channel and a saliva channel. The injected saliva helps to avoid blood coagulation and thus guarantees a constant blood ingestion. The feeding period is rather long reaching (if undisturbed) 20–150 min. The injected saliva does not only help to avoid blood coagulation but also anaesthetizes the biting site. Only when the flea has redrawn its mouthparts from the skin, the biting site starts to

Table 7.3 Important flea species

Species	Size (mm)	Characteristics	Main hosts
Pulex irritans	♂ 2–2.5 ♀ 4	Absence of combs on the surface	Humans, domestic animals
Ctenocephalidis felis *C. canis*	♂ 2 ♀	1 comb at the head 1 comb at the pronotum	Cats, dogs, humans
Xenopsylla cheopis	♂1.5 ♀ 2.5	Absence of any comb	Rats, mice, humans
Ceratophyllus gallinae	♂3 ♀ 3.5	1 comb at the pronotum	Chicken, humans
Echidnophaga gallinacea	♂1.5 ♀ 2–2.5	No combs, female head enters the skin of the hosts	Chicken, dogs, humans (in tropics)
Tunga penetrans	♂0.5–0.7 ♀ 0.5–10	Female enters skin; only the posterior end remains visible	Humans, many domestic animals, rats

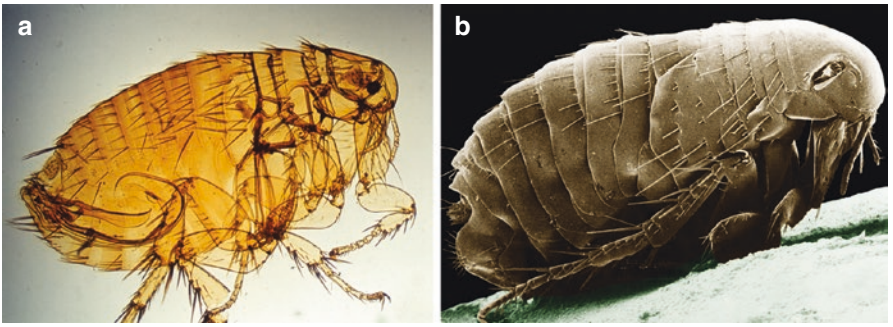

Fig. 7.22 Light microscopic (**a**) and scanning electron micrograph (**b**) of an adult human flea (*Pulex irritans*). Note that there are no combs at the head or along the neck

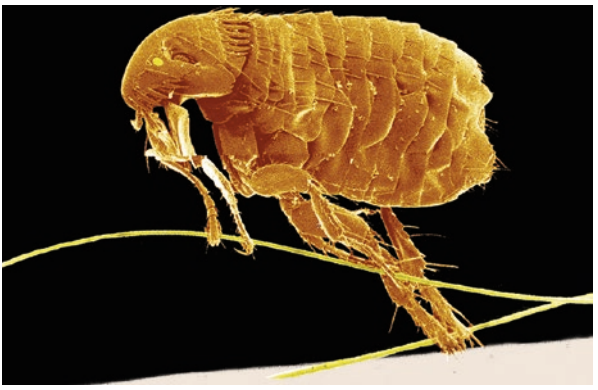

Fig. 7.23 Scanning electron micrograph of an adult female cat flea (*Ctenocephalidis felis*) which is today the most common flea in human dwellings (note the combs of the neck)

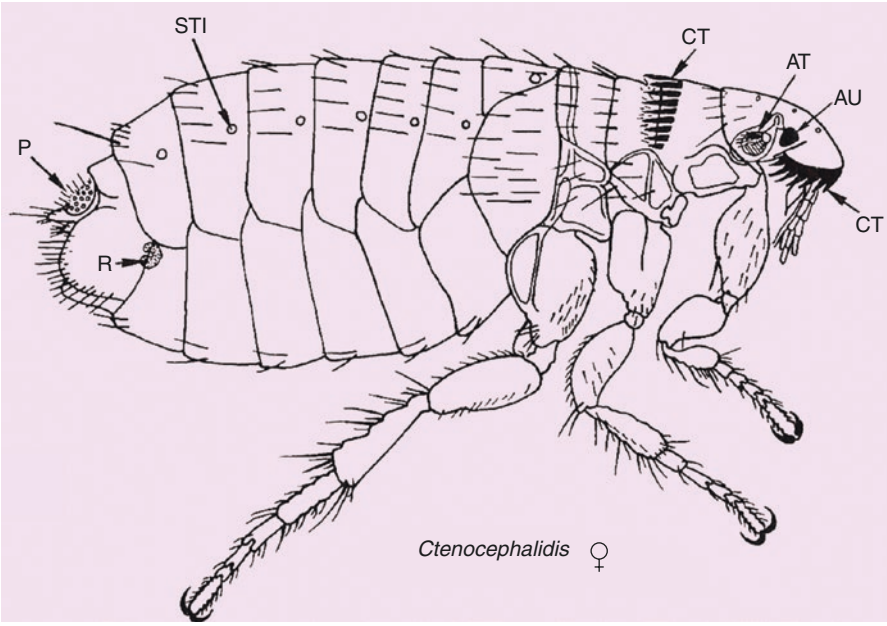

Fig. 7.24 Diagrammatic representation of the lateral aspect of a female cat flea (*C. felis*). *AT* antenna retracted in a groove; *AU* eye; *CT* ctenidia, combs; *P* pygidial plate with sensillae; *R* receptaculum seminis (for sperm storage); *STI* stigma (opening of the tracheal system for air uptake)

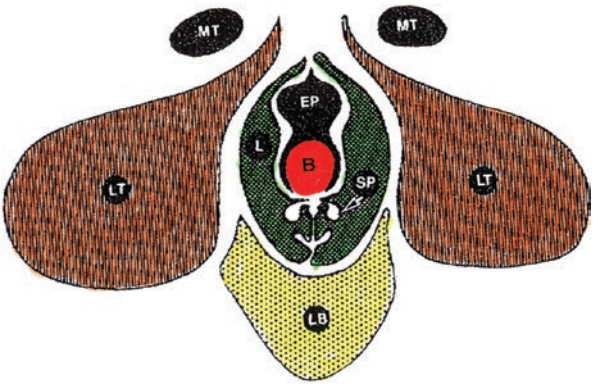

Fig. 7.25 Diagrammatic representation of a cross section through the mouthparts of an adult flea. *B* blood food channel; *EP* epipharynx (forms ventrally the food channel); *L* lacinae, they form each a saliva channel; *LB* labium; *LT* labial sensilla; *MT* maxillary sensilla; *SP* saliva channel

develop an often very intense itching. Since fleas can easily be disturbed during their blood meal, they retract their mouthparts (Fig. 7.25) from the skin and start again at other places of the skin, so that single fleas may induce several itching sites (often in a row) which may react all at the same time, if one person/animal scratches at one place.

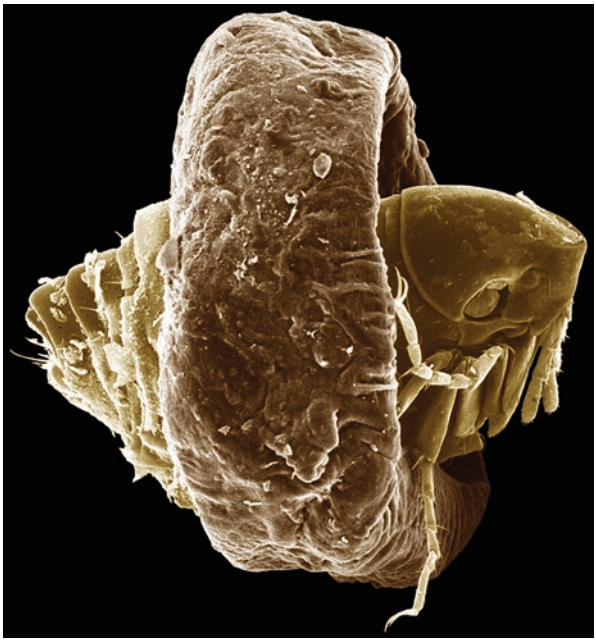

Fig. 7.26 Scanning electron micrograph of a female *Tunga penetrans* sand flea showing its swollen central region (due to a giant egg production). This female was taken out of the skin of a child

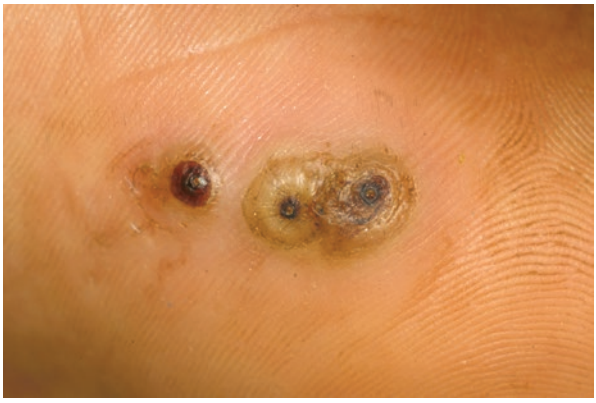

Fig. 7.27 Macrophoto of three swollen female specimens of the so-called sand flea *Tunga penetrans* sticking deeply in the skin of the feet of a child in South America. This region of the fleas contains the openings of the breathing system, the anus, and the ovaries

Experimental transmission studies of our group together with scientists of Bayer Company (Germany) showed that fleas can transmit several bacteria and viruses via excreted feces or via injection of blood-contaminated mouthparts (Mencke et al. 2009; Vobis et al. 2003). Thus the fleas are not only nasty bloodsuckers but also of high importance as potential vectors of agents of diseases, which especially in camps of refugees or crowded townships may induce heavy epidemics, which afford huge efforts to control them.

7.3.2.3 Black Flies: Simuliids

The mostly blackish appearing adults of the genus *Simulium* reach a length of 2–5 mm and thus are mostly considerably smaller than the typical mosquitoes (Culicidae). Exclusively the females (Fig. 7.28) suck blood, while males feed on plant juices. The males are characterized by the fact that their eyes possess two types of lenses in their compound eyes (Fig. 7.29). The mouthparts of the females belong to the sawing type (Figs. 7.29 and 7.30). By the help of their mouthparts,

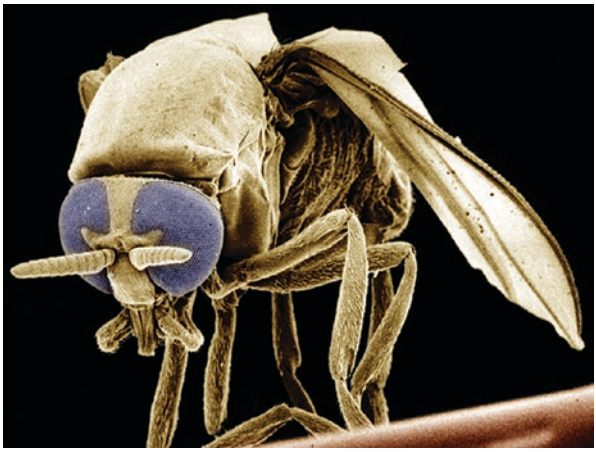

Fig. 7.28 Scanning electron micrograph of a female of the blackfly *Simulium damnosum*

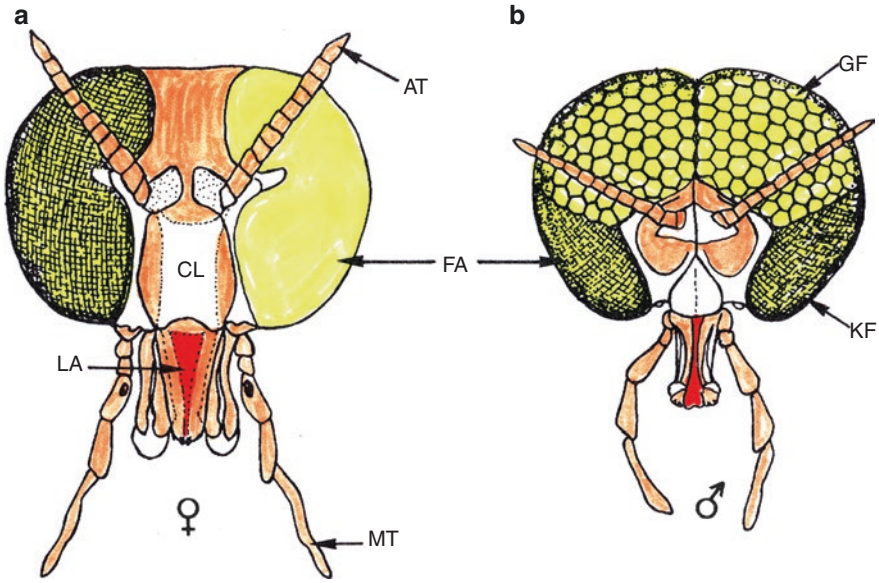

Fig. 7.29 Diagrammatic representation of the heads of female (**a**) and male (**b**) simuliids. *AT* antenna; *CL* clypeus; *FA* facettes of eyes (small and large ones); *GF* large facettes; *KF* small facettes; *LA* labrum; *MT* maxillary sensilla

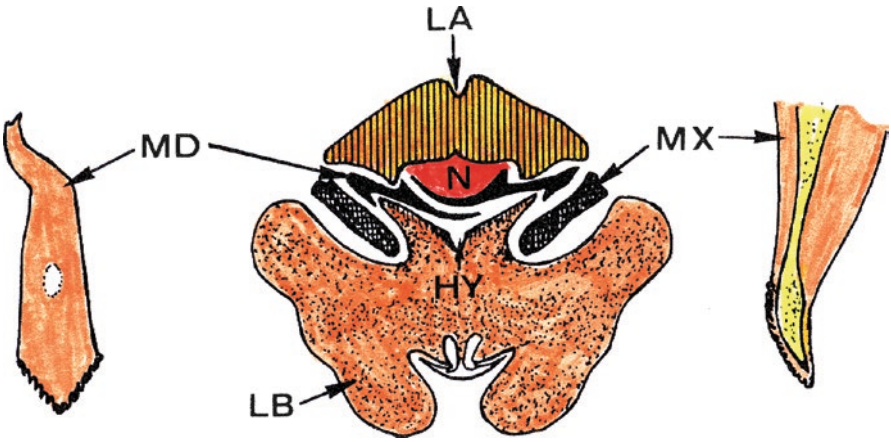

Fig. 7.30 Diagrammatic representation of a cross section through the sawing mouthparts of female simuliids. *HY* hypostome; *LA* labrum; *LB* labium; *MD* mandible; *MX* maxillary sensilla; *N* food channel

they produce little "blood lakes" in the skin of their hosts and thus are described as "pool feeder." This behavior leads to considerable pain so that cattle often flee from attacking black flies, especially during attacks of hundreds of specimen. Four to five days after a blood meal, fertilized females depone up to 250 eggs at plants in quickly running waters. The larvae hatch already after 4 h. The further development takes only 8–9 days, so that in the tropics or during warm European summers, large amounts of these nasty biters attack humans and their animals. In the tropics (Africa, Central America) simuliids transmit the larvae of the worm *Onchocerca volvulus*, which may lead to the so-called river blindness of humans, which, however, is only transmitted by rather few *Simulium* species (e.g., *S. damnosum* and *S. neavei* in Africa and *S. ochraceum*, *S. metallicum*, and *S. callidum* in Central America).

7.3.2.4 Sand Flies: Phlebotomidae

The species of the genus *Phlebotomus* reach mostly only 2.5 mm in length (Fig. 7.31) and are characterized by a dense layer of hair. The females suck blood during the night at human and animal hosts. Thereby they may transmit agents of diseases like the viruses of the pappataci fever, Rickettsiales/bacteria (bartonellosis), and parasites (leishmaniasis of humans and dogs, e.g., in regions around the Mediterranean Sea).

Fig. 7.31 Macrophoto of an adult sand fly (*Phlebotomus* sp.)

7.3.2.5 Mosquitoes: Culicidae

The mosquitoes (in a narrow sense) include the very important genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Mansonia*. Their females suck blood at vertebrates by the help of a tiny sucking apparatus, which contains two channels: one which introduces saliva keeping the host blood fluid and another one through which host blood is engorged (Figs. 7.32 and 7.33). The females of the family Culicidae are able to transmit a broad spectrum of agents of disease (Table 7.4).

7.3.2.6 Tsetse Flies: Glossinidae

The so-called tsetse flies (genus *Glossina*) occur in Africa and are characterized by their appearance, whereby they stretch their mouthparts horizontally forward from their head (Figs. 7.34 and 7.35). The smaller species (*Glossina tachinoides*) reach a length of 6–8 mm, while the larger ones (*G. palpalis*, *G. morsitans*) may even reach

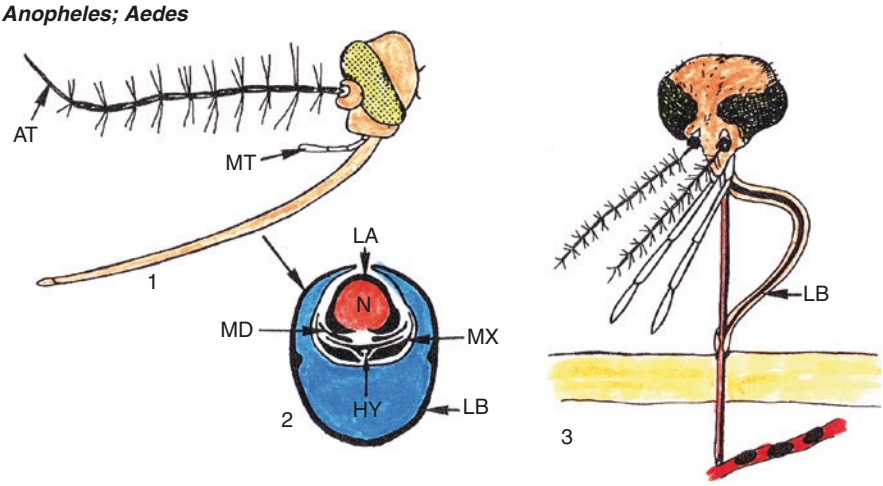

Fig. 7.32 Diagrammatic representation of the head and mouthparts of culicid mosquitoes (e.g., genera *Aedes* and *Anopheles*), where the females are always equipped with two antennae each with 15 segments, while males have only 14 segments. (**1**) Head: equipped with antenna, maxillary sensilla (MT), and labium (LB) enclosing the food and saliva channels. (**2**) Cross section of labium (LB). (**3**) Head and annexes and sucking system injected into a blood vessel inside the skin of a host. *AT* antenna; *HY* hypopharynx with saliva channel; *LA* labrum; *LB* labium (cover of mouthparts); *MD* mandibles; *MT* maxillary sensilla; *MX* maxilla; *N* food channel through which host blood is engorged

a size of up to 9–14 mm. Both sexes suck blood by the help of their large mouthparts, which produce little lacunae in the skin, which become filled with blood. Thus they are so-called pool feeders like simuliids and ticks. The females of the tsetse flies give birth to only one larva at a time and repeat this process about 8–9 times during their about 90-day-lasting life as adults. The tsetse flies transmit in Africa the agents of the so-called sleeping sickness of animals and humans. Especially the latter pay even today a high death toll, since the available medicaments are not very effective and in addition rather toxic.

7.3.2.7 Tabanids

The adults of these rather large (reaching up to 3 cm in length) insects are equipped with saw-like mouthparts, by which the females cut hollows into the skin of animals and humans and suck excreting blood (Figs. 7.36 and 7.37). Due to the large size of the mouthparts, their bites are rather painful and can be easily contaminated by agents of disease, which then may become transmitted mechanically to other hosts. Especially the species of the genus *Chrysops* are able to transmit (in Africa) the filarial worm *Loa loa*, which may pass the eye during their migration inside the body of humans. Besides the rather rare cases of transmission of agents of diseases, the tabanids are frightful due to their painful bites, e.g., by the so-called "rain biting fly" *Haematopota pluvialis* (Fig. 7.37) and related species.

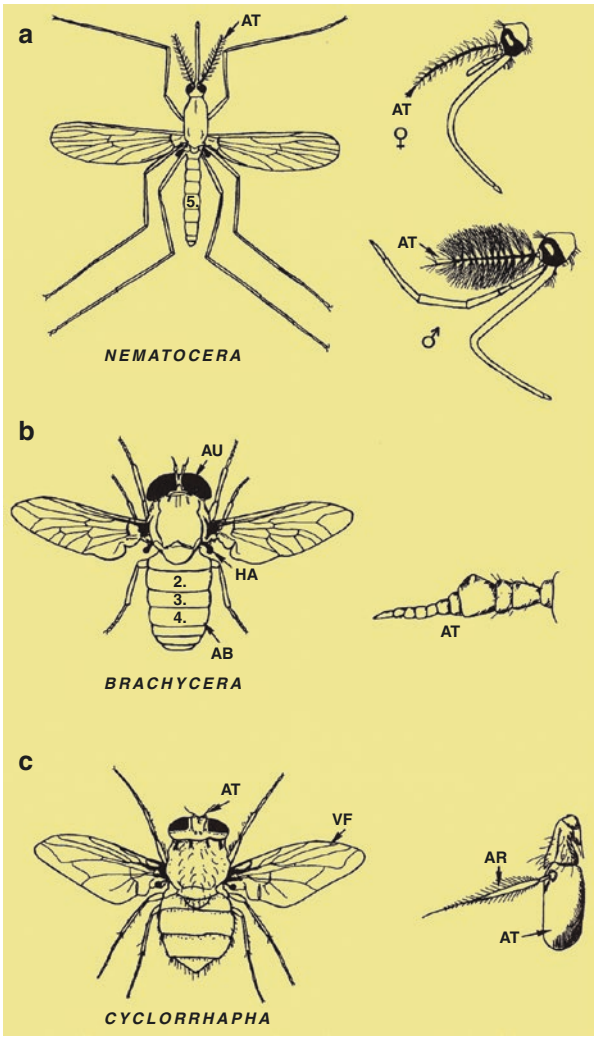

Fig. 7.33 Diagrammatic representation of the characteristics of (**a**) Nematocera, (**b**) Brachycera and (**c**) Cyclorrhapha. *AR* arista; *AT* antennae; *AU* facettes of compound eyes; *HA* halteres; *MT* maxillary palps; *R* sucking channel includes saliva and food channels

Table 7.4 Genera of Culicidae and important transmitted agents of diseases (examples)

Genus	Disease of humans	Agent of disease	Disease of animals	Agent of disease
Aedes	Yellow fever Dengue fever	V V	Rabbit myxomatosis	V
Culex	St. Louis encephalitis	V	Horse encephalitis Chicken malaria	V P
Anopheles	Malaria	P	Malaria	P
All three genera	Filariasis	N	Filariasis	N

N nematode, *P* protozoa, *V* virus

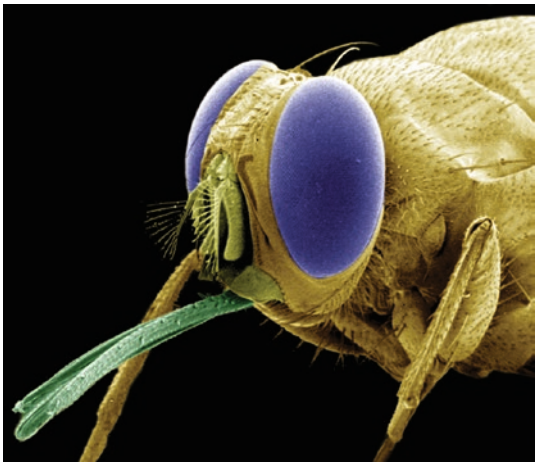

Fig. 7.34 Scanning electron micrograph of an adult tsetse fly (*Glossina morsitans*). Note the typical position of the mouthparts

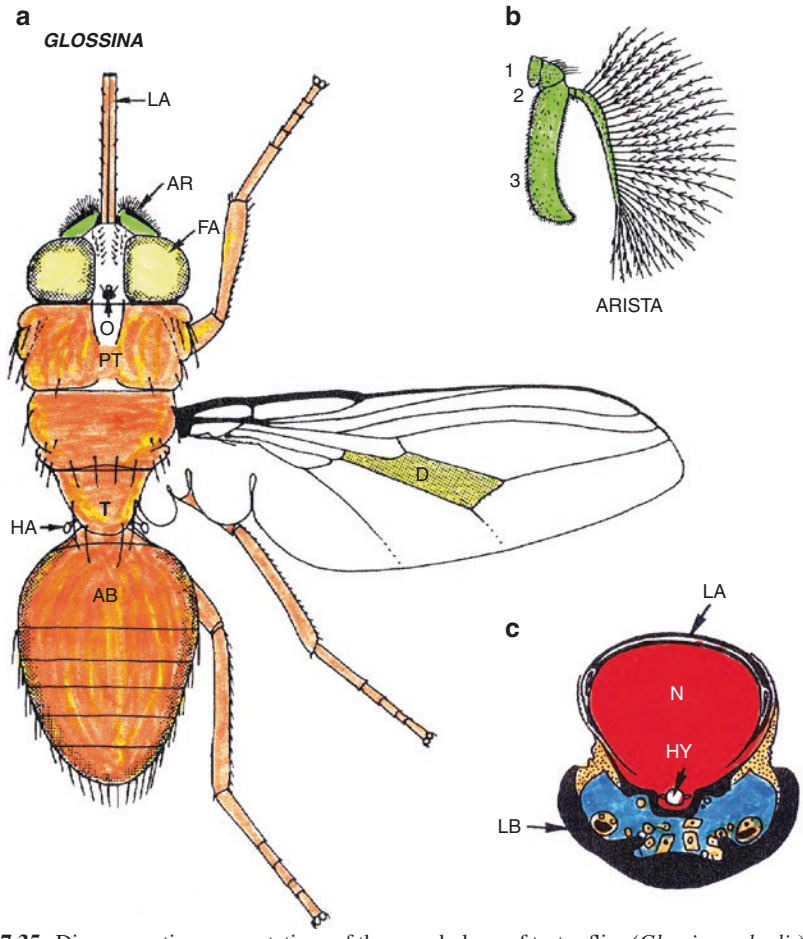

Fig. 7.35 Diagrammatic representations of the morphology of tsetse flies (*Glossina palpalis*). (**a**) Aspect of the dorsal side. (**b**) Antenna (with arista). (**c**) Cross section of mouthparts. *AB* abdomen; *AR* arista; *D* discoidal field; *FA* facette eyes; *HA* halteres; *HY* hypopharynx; *LA* labium; LB labrum; *MT* mesothorax; *N* food channel; *O* ocellus; *PT* prothorax; *T* metathorax

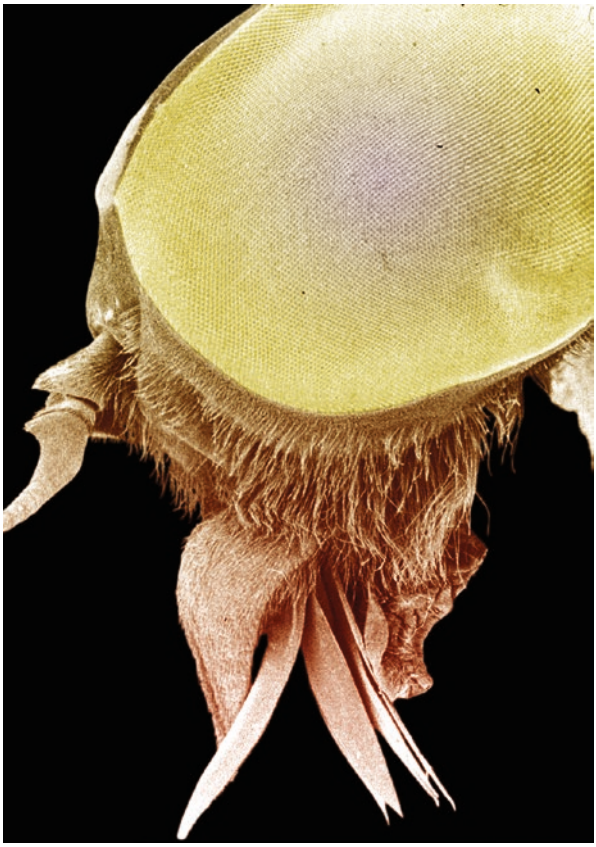

Fig. 7.36 Macrophoto of the head of *Tabanus* sp

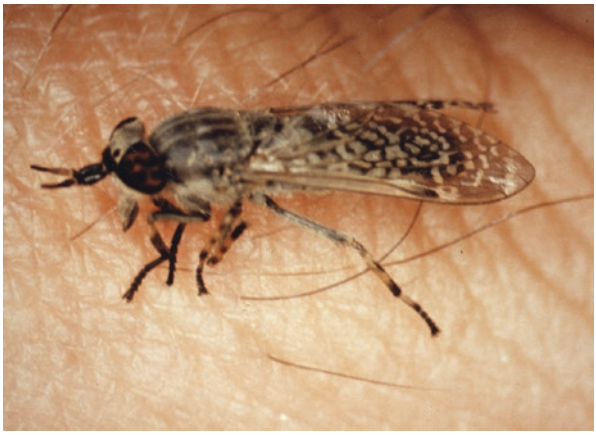

Fig. 7.37 Macrophoto of *Haematopota pluvialis*

7.3.2.8 Ceratopogonidae: Midges

These very tiny (1–4 mm long) mosquito-like insects (Figs. 7.38 and 7.39) endanger especially farmers in tropic countries, but also in Europe, since they transmit viruses (e.g., the bluetongue virus in Europe, African horse sickness virus, and other

Fig. 7.38 Macrophoto of an adult female of *Culicoides obsoletus*, which transmitted in Germany (2006–2009) the bluetongue virus serotype 8 in a broad epidemic

Fig. 7.39 Macrophoto of the wings of *Culicoides* species, which can be used for species identification

arboviruses). Only the females suck blood starting their blood meals mainly in the hours of early evening. The bites induce a feeling of burning, since they possess saw-like mouthparts. The larvae live often also in stables, where they stay in food remnants of cattle.

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Chapter 8
Zika Virus Epidemics: Only a Sudden Outbreak?

Heinz Mehlhorn

Abstract The so-called Zika virus was first described in the year 1947 in a monkey in Uganda. The symptoms in monkeys and members of the local human population had been described as smooth at this time. Thus the presence of the virus and its effects were neglected or underestimated, although larger outbreaks among humans started to occur in 2007 in countries of Micronesia. However, beginning from 2013 an intense spreading started (or at least its notice was taken) in countries of the Pacific region. Special consideration of Zika virus infection was taken during the 2014 Olympic Games in Brazil, when large numbers of children were born showing the severe symptoms of a microcephalus. Today the virus is present in at least 40 countries including especially Central and South America as well as in Florida. Visitors of the Soccer World Championship (2014) and Olympic Games (2016) were apparently infected, too, since many returning Europeans were found to be seropositive for this virus. Although tropical *Aedes* species are constantly imported to European countries, local infection risks remain low, since their propagation at European temperatures is extremely low.

Keywords Zika virus · Outbreaks · Virus epidemics · Malformation · Microcephalus

8.1 History

The so-called Zika virus being named according to a forest region in Uganda (Africa) was first detected in a monkey in Uganda in Central Africa and described in detail by Dick (1952) and Dick et al. (1952). Human cases of illness due to this virus were first reported in the year 1954 during an outbreak of jaundice in Nigeria (Macnamara 1954). Starting from that time until the early years in the 2000s, very few other benign cases of human Zika infections have been described in several countries in Africa and Asia (Indonesia) (Song et al. 2017). The first larger outbreak of the Zika virus out of Africa occurred among inhabitants of the Yap Island, which belongs to the Federated States of Micronesia being situated in the northwestern

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Pacific Ocean. This outbreak involved nearly three quarters of the island population, but the patients showed only rather smooth symptoms of disease such as fever, rash, arthralgia, and conjunctivitis (Lanciotti et al. 2008; Duffy et al. 2009). During the following years, only sporadic cases of Zika virus infections occurred in Southeast Asian countries (e.g., Thailand, Cambodia, Indonesia, Malaysia, the Philippines) (Song et al. 2017) followed by numerous cases in French Polynesia (Mallet et al. 2015), where, however, several persons were severely hit by neurological symptoms such as the so-called Guillain-Barré syndrome as co-symptoms to chikungunya and dengue fever (Oehler et al. 2014). In the following years, human cases occurred in many countries like Australia, Italy, Japan, Norway, etc. (Song et al. 2017; Possas 2016; Ali et al. 2017; Waddel and Greig 2016). At the beginning of the year 2015, many cases of Zika virus infections were detected in Brazil and increased in numbers of up to 1.3 million cases including an extremely large number of at least 4000 cases of microcephalus formations in newborn babies (Hennessey et al. 2016). During international events (Soccer World Championship, Olympic Games) in Brazil, numerous visitors apparently became infected and imported this virus to their countries, where it—at least up to now (2018)—is not spreading yet due to the still rather low number of relevant *Aedes* species and low temperatures.

8.2 Symptoms of Disease

The general symptoms of an infection by the Zika virus are similar to those of the dengue fever but are mostly less intensive. Common symptoms are rash, headache, muscle pain, inflammation of the eyes, and fever. These symptoms last for 3–14 days (3–7) after humans have been infected during a mosquito bite and last mostly 1 week. In some rather rare cases also, a Guillain-Barré syndrome was described. The clinical features of dengue, Zika, and chikungunya were compared by Ali et al. (2017) (Table 8.1).

Table 8.1 Comparison of Dengue, Zika and Chikungunya symptoms

Clinical features	Dengue	Zika	Chikungunya
Onset postinfection	(4–7) d	1–5 become ill	(3–7) d
Fever	>38 °C	None or mild fever	High fever >38 °C
Headache	Very common	Common	Common
Rash	Common	Very common	Very common
Itch	Common	Common	Common
Joint pain (arthralgias)	Yes	Common	Very common
Muscle pain (myalgias)	More common	Common	Common
Red eye (conjunctivitis)	None	Very common	Yes/none
Thrombocytopenia	Very common	Less common	None
Low level of blood cells and platelets	Very common	None	Common
Bleeding disorder	Very common	None	Yes/none
Shock	Yes/none	None	None
Recovery of low symptoms	6–7 d	4–7 d	<1 week

As a recent Zika complication—especially in Brazilian cases—more than 4000 newborn babies were born after the year 2014 with a so-called microcephalus. This incidence is 20-fold higher than in the preceding years 2010–2014 and is not yet completely understood. Also ocular abnormalities were frequently found in these newborns as well as acute myelitis (Ali et al. 2017).

8.3 Zika Virus Genome

This flavivirus has a single-stranded positive-sense RNA genome with a length of about 10.7 kb being enclosed in a capsid and surrounded by a membrane. About 13 recombination events have been reported from the primary analysis using sequencing tool RDP (Recombination Detection Project, Brown et al. 2016).

8.4 Vectors

The Zika virus is transmitted in the tropical and subtropical regions by the mosquito *Aedes aegypti*. Recent papers also clearly indicated that also *Aedes albopictus* (tiger mosquito, Fig. 8.1) is able to transmit this virus. Since this virus is actually invading Europe, Zika viruses might be transmitted soon in Europe, too, as soon as sufficient mosquitoes are present as well as sufficient infected humans come back from endemic countries. The newsletter of the Robert Koch Institute at Berlin also reported cases of sexual transmission occurring apparently even weeks after returning from endemic countries.

Fig. 8.1 Macrophoto of a typical female *Aedes* specimen. The species of the whole genus are characterized by more or less large white dots along the body and along the legs. Since in some species these dots are large, they got the trivial name "tiger mosquito"

8.5 Therapy

Until today (2018) there exist neither vaccines nor medicaments which could be used for protection respectively for treatment.

8.6 Occurrence and Protection

In Germany up to now, about 300 low-graded infections have been noted in persons returning from South America. Like in other countries, Zika virus infections have to be obligatorily announced in Germany to the health authorities. Thus tourists and workers entering endemic regions should protect themselves by consequent use of repellent substances (Icaridin, DEET, IR3535) from bites of the mosquitoes of the genus *Aedes*, which are active during daytime.

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Chapter 9
Mosquitoes as Arbovirus Vectors: From Species Identification to Vector Competence

Claudia Schulz and Stefanie Christine Becker

Abstract Mosquitoes and other arthropods transmit a large number of medically important pathogens, in particular viruses. These arthropod-borne viruses (arboviruses) include a wide variety of RNA viruses belonging to the *Flaviviridae* family (*West Nile virus* (WNV), *Usutu virus* (USUV), *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), *Zika virus* (ZIKV)), the *Togaviridae* family (*Chikungunya virus* (CHIKV)), and *Bunyavirales* order (*Rift Valley fever virus* (RVFV)) (please refer also to Table 9.1). Arboviral transmission to humans and livestock constitutes a major threat to public health and economy as illustrated by the emergence of ZIKV in the Americas, RVFV outbreaks in Africa, and the worldwide outbreaks of DENV. To answer the question if those viral pathogens also pose a risk to Europe, we need to first answer the key questions (summarized in Fig. 9.1):

1. **Who** could contribute to such an outbreak? Information about mosquito species resident or imported, potential hosts and viruses able to infect vectors and hosts in Germany is needed.
2. **Where** would competent mosquito species meet favorable conditions for transmission? Information on the minimum requirements for efficient replication of the virus in a given vector species and subsequent transmission is needed.
3. **How** do viruses and vectors interact to facilitate transmission? Information on the vector immunity, vector physiology, vector genetics, and vector microbiomes is needed.

Keywords Zoonotic arbovirus · Europe experimental infection · Vector competence · Antiviral immune response taxonomy

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9.1 Who Could Contribute to an Arbovirus Outbreak

9.1.1 Taxonomy and Mosquito Surveillance in Europe

As the spread of mosquito-borne arboviruses is dependent on the presence of a suitable mosquito vector, the knowledge of the mosquito species distribution and vector competence of these mosquitoes belongs to the most crucial factors for estimations about the risk of mosquito-virus emergence to new areas or maintenance of (endemic) arboviruses within particular regions. The first critical issue for mosquito surveillance programs is the exact classification of species (Fig. 9.1).

To facilitate detection of different species, several methods have been proposed. Classical morphology is used as the first line of classification. Several keys for morphological discrimination have been published. The morphological characteristics described by Mohrig (1969) and Becker et al. (2010) have been most commonly used for species identification in surveillance programs in Germany. These programs include several university- and organization-driven approaches, some as a part of the European project VBORNET (http://www.vbornet.eu/) or the citizen science project "Mückenatlas" (Walther and Kampen 2017). All those projects have made large progress in redefining the mosquito fauna in Europe and Germany. Especially the "Mückenatlas" project has also proven a very sensitive tool to detect new and invasive species as, for example, several new populations of *Aedes japonicus japonicus* in North Rhine-Westphalia and Lower Saxony and *Aedes albopictus* populations in Baden-Wuerttemberg (Kampen et al. 2016a; Werner and Kampen 2013; Werner et al. 2012; Zielke et al. 2014).

Within all programs, the classical morphology has proven a useful tool. However, the accuracy of classical morphological classification is strongly dependent on expert knowledge and the availability of good-quality mosquito specimens. Furthermore, several cryptic species allow only for classification according to male mosquitoes, which are often not attracted by the traps used for surveillance programs. Especially females of the *Culex pipiens* complex (Fonseca et al. 2004) and the *Anopheles maculipennis* complex (Kronefeld et al. 2012, 2014; Proft et al. 1999) turned out to be difficult or impossible to distinguish in case of morphologically similar sibling species, such as *Culex torrentium* and the two *Culex pipiens pipiens* biotypes *pipiens* and *molestus* or mosquito species belonging to the *Anopheles messeae/daciae* complex. Both species complexes are of major importance for disease transmission: *Culex pipiens* a main vector for WNV, USUV, or RVFV and *Anopheles maculipennis* as a potential vector for *Plasmodium* parasites. Hence, classification methods besides morphology are needed to reach a satisfactory level of species discrimination (Bickford et al. 2007).

The use of morphometric analysis as a qualitative tool for species discrimination has expanded during the past years (reviewed by Lorenz et al. (2017)). In particular wing shape has been used for morphometric comparison in mosquito studies. Wilke et al. (2016) have established a protocol for geometric wing morphometries to identify a broad range of medically important mosquito species belonging to the *Aedes*, *Culex*, and *Anopheles* genera. To do so, 18 landmarks at wing vein intersections

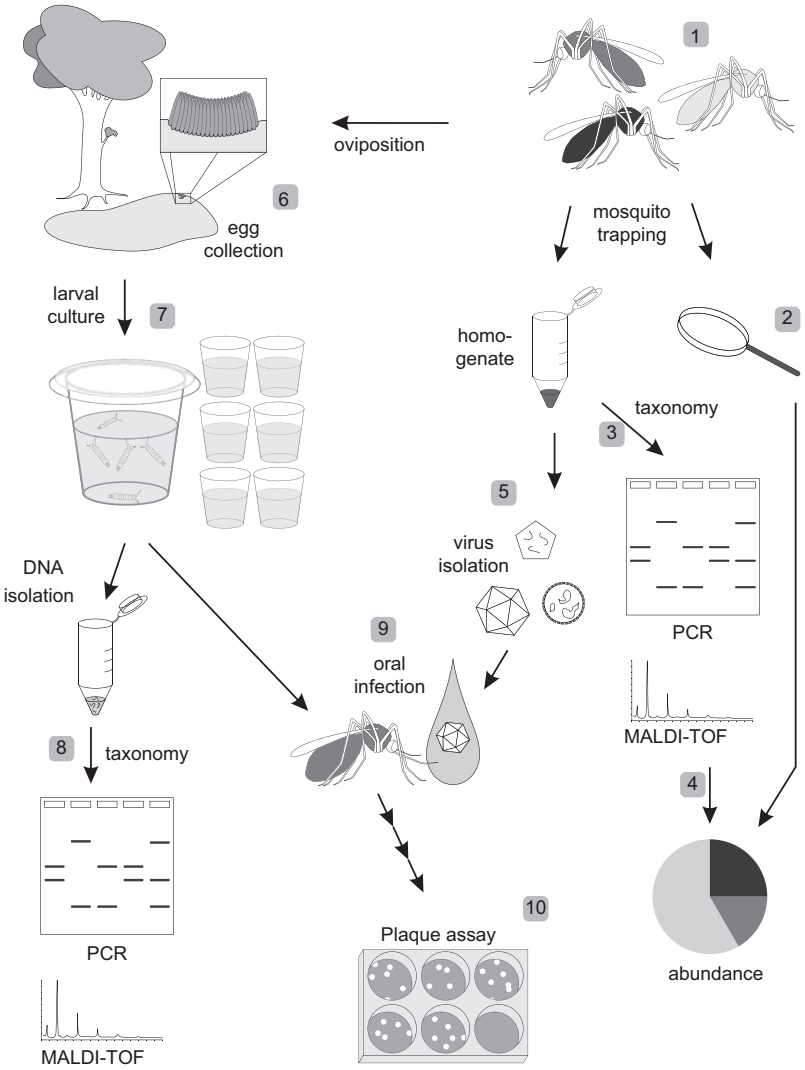

Fig. 9.1 Graphical representation of vector competence assay. The analysis of resident mosquito populations for virus presence and vector competence for the respective virus starts with the collection of mosquitoes (1). Subsequently, the mosquitoes are subjected to morphological taxonomic classification (2) and are pooled according to species and location. Mosquito pools are homogenized to isolate nucleic acids for PCR and proteins for MALD-TOF MS. These data are used for taxonomic confirmation (3) and abundance statistics (4) or virus screening. Virus-positive pools will be used for virus isolation (5) which can then be used for vector competence assays via oral infection (9). To obtain mosquito samples for vector competence assays, eggs of resident mosquito populations are collected (6) and reared in the laboratory (7). From each larval culture, some specimens will be used for taxonomic identification (8). Larvae from the same location and species are pooled, and emerging adult females will be used for vector competence assays. New virus isolates are mixed with blood and fed to 4–7-day-old female mosquitoes (9). After different times of infection, some mosquitoes are sacrificed, and body infection rates (IR), dissemination rates (DR), and transmission rates (TR) will be measured by virus titration (10)

were collected from digitalized photographs of female wings. Mosquito genera were classified with 99% accuracy and species even with 100% accuracy, demonstrating the power of the approach (Wilke et al. 2016).

Several other groups also used this method to discriminate female samples of closely related cryptic species. Lorenz et al. (2012) analyzed the same 18 landmarks to distinguish between *Anopheles cruzi, Anopheles homunculus*, and *Anopheles bellator* mosquitoes and reached 78–88% accuracy. For the *Culex* complex, differences in wing venation were already described by Natvig (1948) and Mohrig (1969), who also proposed to use these differences for species discrimination. Especially the vein R2/3 was found informative for differentiation of *Culex pipiens* and *Culex torrentium* females. Borstler et al. (2014) used general wing morphology and the R2/3 indices for discrimination of *Culex pipiens* and *Culex torrentium* collected in Germany. Their study revealed more than 91% accuracy in the multivariant morphometric analysis using several wing landmarks and 90% correct species identification when only using the R2/3 vein indices. Thus, the morphometric discrimination method has been proven to be a stable and reliable method with success rates of 70–100% for correct reclassification (Lorenz et al. 2017). It is particularly tempting that this morphometric method has been shown to be most accurate in female mosquitoes, the main object of interest in the context of vector-borne diseases.

Although geometric morphometry is a quick and easy to use method, it should be noted that data capturing and identification of landmarks are still a critical issue. Furthermore, in large-scale surveillance programs, a certain degree of automatization of landmark detection and automatic species identification needs to be made, in order to ensure a timely species identification (Lorenz et al. 2017). Thus, molecular methods for large-scale species identification are still needed. In recent years, several advances in the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been explored to achieve species differentiation. MALDI-TOF MS has been extensively used in bacterial diagnostics (Dierig et al. 2015) and for species identification of *Drosophila* (Feltens et al. 2010) as well as of relevant vector species such as *Culicoides* biting midges (Kaufmann et al. 2012), *Phlebotomus* sand flies (Mathis et al. 2015), and *Ixodes* ticks (Yssouf et al. 2013a, 2015). Due to the extensive use in diagnostics, a lot of laboratories adjacent to clinics have already implemented MALDI-TOF MS facilities that can easily be used for mosquito surveillance programs. The adaptation of the MALDI-TOF MS for mosquito species identification has made great advances in the past years. Yssouf et al. (2013b) described this technique to analyze samples from tropical areas and were able to establish profiles from 20 mosquito species collected in La Réunion Island and Senegal. In this study, a reliable classification on subspecies level was achieved as demonstrated for the M and S forms of *Anopheles gambiae.* In total, 100% of the samples were identified correctly after generation of a spectra database. Therefore, this score was set a cutoff value for species identification. However, the method was not considered suitable for mosquito phylogeny yet. To refine the database and to add new species to the collection, Yssouf et al. (2014) conducted a subsequent study using mosquito samples of 11 different species collected at different sites in France and Sweden. After the generation of reference

samples based on previous morphological characterization (Becker et al. 2010), 88.5% of the samples were identified correctly. These and other studies (Raharimalala et al. 2017; Schaffner et al. 2014) showed the feasibility and reliability of MALDI-TOF MS for mosquito species identification. Furthermore, the method is usually described as an inexpensive and easily implementable approach. However, a certain degree of instability was recently detected in mosquito samples collected in different countries, highlighting the importance to establish an international database to assure correct mosquito species identification.

Another very sensitive and reliable method for species identification and the differentiation of cryptic species or biotypes is their genetic characterization by conventional and real-time PCR using phylogenetic markers: either chromosomal markers such as the acetylcholinesterase 2 (*ace2*) gene, the second internal transcribed spacer (ITS2), and the microsatellite locus *CQ11* or mitochondrial barcoding based on the cytochrome oxidase I (*COI*) gene. The use of the *ace2* locus as a diagnostic criterion for the differentiation of *Culex pipiens* complex (*Culex pipiens*, *Culex quinquefasciatus*, *Culex p. pallens*, *Culex australicus*), *Culex torrentium*, and *Culex pervigilans* mosquitoes was developed by Smith and Fonseca (2004). Mosquitoes of these species were collected across the world and subjected to PCR analysis using diagnostic primers located between exons 2 and 3 of the *ace2* genetic locus. The *ace2* PCR assay was able to distinguish and to detect hybridization events between the mentioned *Culex* species, for example, hybridization of *Culex quinquefasciatus* and *Culex pipiens*, but the two bioforms *Culex pipiens pipiens* biotype *pipiens* and *Culex pipiens pipiens* biotype *molestus* could not be discriminated. The two biotypes show different feeding patterns and (breeding) habitat preferences with *Culex pipiens pipiens* biotype *molestus* being more anthropophilic and adapted to urban habitats, whereas the biotype *pipiens* is more ornithophilic and adapted to a wide range of natural habitats. These differences in lifestyle can have major impact on their ability to act as vectors for viruses such as WNV. Indeed, *Culex pipiens pipiens* biotype hybrids have been widely discussed as potential bridge vectors between birds and humans for bird-associated viruses such as WNV. Thus, the correct identification of these two biotypes can be crucial for correct risk assessment. To improve biotype differentiation, Bahnck and Fonseca sequenced microsatellite loci of the *Culex pipiens* complex and found that the *CQ11* locus was suitable for the diagnosis and differentiation of two *Culex pipiens* biotypes (Bahnck and Fonseca 2006).

The two assays *ace2* and *CQ11* (Bahnck and Fonseca 2006; Smith and Fonseca 2004) were used to design a multiplex qPCR assay which allows the differentiation of *Culex* species, biotypes, and biotype hybrids within one reaction (Rudolf et al. 2013). Using a large collection of about 349 morphologically well-defined mosquito specimens (consisting of 227 *Culex pipiens* biotype *pipiens*, 3 *Culex pipiens* biotype *molestus*, and 119 *Culex torrentium* samples), the assay was evaluated and revealed 100% specificity for the respective *Culex* species or biotypes (Rudolf et al. 2013). The analysis of 16,566 *Culex* samples collected at different trapping sites in Germany with this multiplex qPCR revealed that *Culex pipiens* biotype hybrids are also present in Germany. Furthermore, the expansion of *Culex torrentium* in Central

Europe was confirmed with more than 50% of the collected specimens containing *Culex torrentium* at some sample locations in Germany. The same multiplex qPCR method was also used for a surveillance study in the Emilia-Romagna in Italy, which revealed that all (100%) of the 24,165 tested mosquitoes were *Culex pipiens* and that *Culex torrentium* was absent at these sample locations (Calzolari et al. 2016). This is in agreement with other studies performed across Europe by Hesson et al. (2014) analyzing 2559 larval samples from 138 collection sites in 13 European countries. This study found *Culex torrentium* more prevalent than *Culex pipiens* in Central and Northern Europe but mostly absent in Southern Europe. The study by Hesson et al. (2014) used a different method based on the amplification of the 3′-end of the *COI* locus, subsequent restriction digest, and sequencing (Hesson et al. 2010) for genetic characterization of *Culex pipiens* and *Culex torrentium*. The mitochondrial *COI* gene is often used for species identification or confirmation of morphological classification. To do so, the 5′ part *COI* gene is amplified with generic primer sets, and the PCR products are usually sequenced and analyzed (Folmer et al. 1994). According to Hebert et al. (2003), this method is adequate to "barcode" most animal species with an intraspecies variation mostly below 2% and thus allows for reliable intraspecies identification. The *COI* barcoding has then been used in a large-scale approach such as the International Barcode of Life (iBOL) project creating a reference database BOLD (www.boldsystems.org). In subsequent years, the method had become a standard technique to identify mosquito species from different countries around the world including China (Wang et al. 2012), Pakistan (Ashfaq et al. 2014), Chile, and Sweden (Engdahl et al. 2014). However, in the Swedish study (Engdahl et al. 2014), some inconsistencies between morphological discrimination and barcoding results were observed. Furthermore, the method may cause inconclusive results in closely related species such as species belonging to the *Culex pipiens* complex. In this case, additional methods such as the restriction analysis of the *COI* PCR fragment described by Hesson et al. (2010) or additional PCRs for the *ace-2* and *CQ11* loci (Bahnck and Fonseca 2006; Fonseca et al. 2004; Rudolf et al. 2013) can be advantageous.

The *Anopheles maculipennis* complex comprises 10–12 Palearctic species (Harbach 2004), and members of the complex have been associated with *Plasmodium*, Sindbis virus, and Batai virus transmission in Europe (Jost et al. 2010, 2011b; Kampen et al. 2016b). In light of the risk for reintroduction of *Plasmodium* species by enhanced global travel, identification of potential malaria vectors is of major interest. Therefore, in 1999, Proft et al. (1999) developed a diagnostic PCR method for identification of the members of this complex that are otherwise indistinguishable. This PCR assay was based on the *ITS2* region, which had been previously used for differentiation of other complexes (Crabtree et al. 1995; Wesson et al. 1992). The PCR products were sequenced, results were compared with morphological classification, and a stable PCR assay for identification of *Anopheles atroparvus*, *Anopheles melanoon*, *Anopheles sacharovi*, *Anopheles maculipennis s. s.*, *Anopheles messeae*, and *Anopheles labranchiae* was established. In the following years, surveillance studies revealed that particularly *Anopheles messeae* is widespread across Central Europe. However, in a study of Novikov and Shechenko in

2001, it became evident that *Anopheles messeae* was not a single species (Novikov and Shevchenko 2001) but represents two cryptic species, *Anopheles messeae* and *Anopheles daciae*, which was confirmed 3 years later (Nicolescu et al. 2004). To differentiate these cryptic species, the *ITS2* assay was refined by the addition of a restriction fragment length polymorphism (RFLP) analysis after *ITS2* amplification (Kronefeld et al. 2012, 2014). Also, Weitzel et al. (2012) refined the *ITS2* analysis to facilitate *Anopheles messeae* and *Anopheles daciae* differentiation by adding a sequencing reaction after initial amplification. However, both protocols are somewhat laborious and prone to contamination. Thus, in 2016, Luhken et al. (2016) described a new multiplex qPCR method to discriminate between the most prominent members of the *Anopheles maculipennis* complex in Central Europe (i.e., *Anopheles maculipennis*, *Anopheles messeae s.l.*, and *Anopheles atroparvus*) and a fluorescence resonance energy transfer (FRET)-based assay to distinguish between *Anopheles messeae s.s* and *Anopheles daciae*. As a result of the large-scale study following the establishment of this method, 1445 mosquitoes from Germany were screened, and the superior spread of *Anopheles messeae* in Central Europe was confirmed with approximately 70% of the samples belonging to this species.

9.1.2 Virus Surveillance in Europe

During the last decade, multiple previously exotic arboviruses that belong to different virus families which may have considerable implications on human and/or animal health have emerged in Europe. Notable examples are mosquito-borne viruses such as CHIKV (*Togaviridae*) and DENV and ZIKV (*Flaviviridae*) as well as *Culicoides*-borne viruses such as *Bluetongue virus* serotype 8 (BTV-8; *Reoviridae*) and *Schmallenberg virus* (SBV, an *Orthobunyavirus* within the *Peribunyaviridae* family and *Bunyavirales* order). Their unexpected emergence—facilitated by globalization and climate change—highlight the risk of future introductions and spread of additional pathogenic arboviruses to Europe such as (1) mosquito-borne *Bunyamwera orthobunyavirus* (BUNV, *Peribunyaviridae*), *O'nyong-nyong virus* (ONNV, *Togaviridae*), (2) mosquito- and *Phlebotomus*-borne RVFV (*Phenuiviridae*), (3) *Culicoides*-borne *Oropouche virus* (OROV, *Peribunyaviridae*), or (4) tick-borne *Crimean-Congo Hemorrhagic fever virus* (CCHFV, *Nairoviridae*) (Amraoui and Failloux 2016; Brustolin et al. 2017; Carpenter et al. 2013; Heitmann et al. 2017; Negredo et al. 2017; Rudolf 2015; Tappe et al. 2014). Importantly, the introduction of novel viruses to regions where related (endemic) viruses circulate can result in reassortment and a consequential change in pathogenicity and phenotype (Briese et al. 2013; Rudolf 2015). A large outbreak of hemorrhagic fever in humans was reported in Africa in the 1990s caused by a reassortant of African strains of BUNV and *Batai virus* (BATV, an infraspecies of BUNV), namely, *Ngari virus* (NRIV) (Gerrard et al. 2004). Repeated introductions of emerging zoonotic mosquito-borne viruses in addition to CHIKV and ZIKV have been reported in Europe, including DENV and *Yellow fever virus* (YFV, *Flaviviridae*) (Húbalek 2008). The detection of

genome fragments of JEV, another flavivirus, in *Culex pipiens* mosquitoes caught in Italy (2010/2011) indicated a repeated introduction or enzootic circulation of JEV or of a related virus in Southern Europe (Cleton et al. 2014; de Wispelaere et al. 2017). A series of repeated disease outbreaks in humans were caused by various zoonotic mosquito-borne viruses endemic or emerging in Europe. In recent years, human virus infections or disease outbreaks were reported in Europe, including chikungunya in Italy (2007) and France (2010, 2014); dengue in Croatia (2010), France (2010, 2013, 2014), and Portugal (Madeira; 2012); usutu in Italy (2009) and Croatia (2013) (Kampen and Werner 2015); and West Nile fever in Austria (2009, 2010, 2014–2016), Croatia (2012–2013), France (2015), Greece (2010–2014), and Italy (2010–2015) (ages 2017; Gossner et al. 2017; Kampen and Werner 2015). WNV is considered endemic in Europe. However, there are several neglected zoonotic arboviruses circulating in Europe that may (at least occasionally) cause disease in humans and animals: BATV, *Tahyna virus* (TAHV, infraspecies of *California encephalitis orthobunyavirus*, *Peribunyaviridae*), SINV (*Alphavirus, Togaviridae*), and *Inkoo virus* (INKV, infraspecies of *California encephalitis orthobunyavirus*) (Eckerle et al. 2018; Húbalek 2008). In general, three groups of mosquito-borne viruses can be distinguished according to their clinical signs: (1) fever-arthralgia-rash (e.g., DENV, CHIKV, ONNV, ZIKV, WNV), (2) affection of the central nervous system (e.g., DENV, ZIKV, WNV), and (3) hemorrhagic fever (e.g., DENV, RVFV) (reviewed by Eckerle et al. (2018); Húbalek (2008); Kampen and Werner (2015)).

9.2 Where Would Competent Mosquito Species Meet Favorable Conditions for Transmission?

9.2.1 Factors for Arbovirus Transmission

Implications for public health emitting from arboviruses depend on key factors that influence vectorial capacity such as barriers to the infection and transmission of arboviruses to mosquitoes (Hardy et al. 1983; Mellor 2009) as well as biotic and abiotic factors that vice versa have an effect on the intrinsic infection barriers. Climate, in particular temperature and precipitation, and ecological factors (in particular land use, anthropogenic disturbance/urbanization) are main abiotic drivers determining the probability of transmission within a given region (Húbalek 2008; Junglen 2016; Kramer 2016). Biotic factors include (1) the susceptibility to infection (e.g., immunogenetics), host diversity, density, behavior, and seasonal abundance of vertebrate hosts (e.g., migration of birds) and their capability to efficiently amplify and transmit the virus to mosquitoes; (2) the vectorial capacity, density, and (opportunistic) feeding preferences concerning the blood source of invertebrate hosts; (3) the genotype and phenotype/pathogenicity of a virus; as well as (4) the interaction, variability, and adaptation between virus genotype x vector genotype and immune system x vertebrate genetics and immune system (Fros et al. 2015a; Kramer 2016; Lambrechts et al. 2009b).

9.2.2 *Barriers to the Infection and Transmission of Arboviruses by Mosquito Vectors*

Vector competence generally depends on intrinsic factors of the mosquito (Hardy et al. 1983) (Fig. 9.2). After the ingestion of an infectious blood meal, the pathogen has to overcome several barriers in the insect host before transmission with injected saliva during a blood meal on a vertebrate host can occur. The midgut infection barrier (MIB) and midgut escape barrier (MEB) may impede virus passing the midgut cells into the hemocoel (Hardy et al. 1983; Mellor 2009; Mellor et al. 2000). An interference of virus dissemination from the fat cells (dissemination barrier, DB) in the hemocoel is explained by the fact that the fat body plays a role in the insect immune response and prevents infection of other tissues (see section innate immunity below (Mellor 2009)). Further barriers include the salivary gland infection

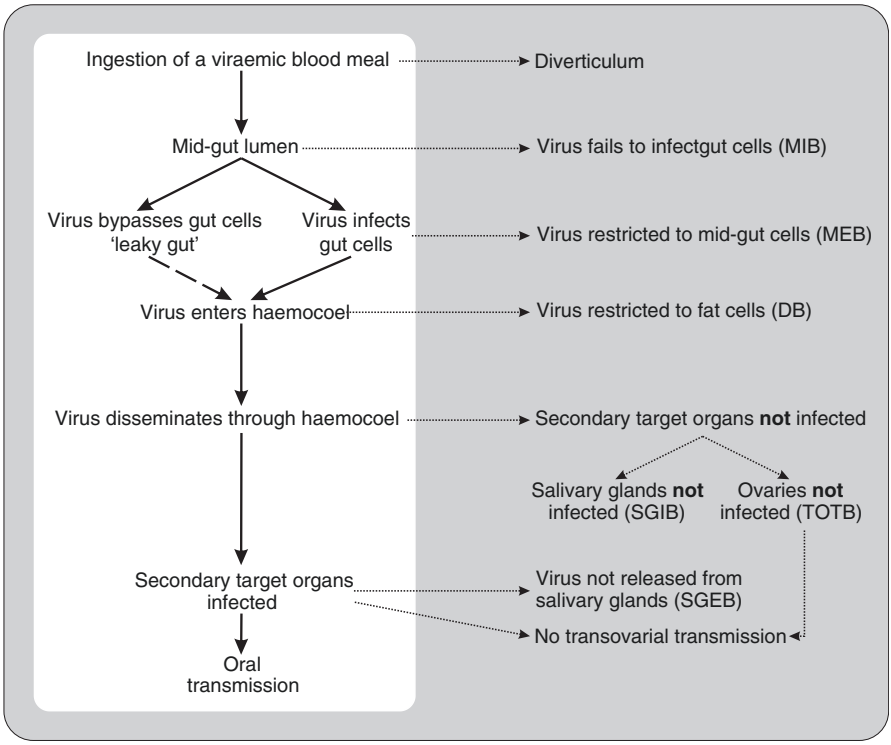

Fig. 9.2 Intrinsic barriers to infection and transmission of arboviruses in mosquitoes. The midgut infection barrier (MIB), midgut escape barrier (MEB), dissemination barrier (DB), salivary gland infection barrier (SGIB), salivary gland escape barrier (SGEB), and transovarial transmission barrier (TOTB) are potentially interfering with infection, dissemination, and transmission of viruses after the ingestion of an infectious blood meal by a mosquito. Only virus release in the saliva and transmission by bite of a vertebrate host confirm the completion of the extrinsic incubation period (EIP) and vector competence of a mosquito (Adapted from Mellor et al. 2000; Hardy et al. 1983)

barrier (SGIB), salivary gland escape barrier (SGEB), and transovarial transmission barrier (TOTB) (summarized in Fig. 9.2). Mosquito females that survive the extrinsic incubation period (EIP, the interval between ingestion of a virus and the earliest time at which virus is released in saliva) potentially remain infectious throughout their life (Hardy et al. 1983; Mellor 2009; Mellor et al. 2000). Experimental vector competence studies regularly include analysis of the infection rate (IR), dissemination rate (DR), and transmission rate (TR). Due to the various possible barriers of an insect host, the TR (defined as the number of mosquitoes with virus-positive saliva per number of virus-positive mosquito bodies (Heitmann et al. 2017)) provides the most important information about the vector competence of a mosquito since only virus transmission by saliva during an insect bite is infectious for the vertebrate host. On the other hand, differences in the IR, DR, and TR may give useful information about possible barriers for a certain virus within an insect host. Furthermore, the EIP depends on the invertebrate host-virus interaction and on the ambient temperature (Mellor 2009) (Table 9.1). Mosquitoes that are not (typical) vectors for a given virus may get competent if reared at elevated temperatures, as reported for *Culicoides nubeculosus* biting midges (a potential vector of BTV). A possible reason is that an increased temperature during the immature stage of the mosquito may compromise the integrity of the gut wall enabling virus to bypass the gut barrier ("leaky gut" phenomenon) (Wittmann and Baylis 2000). In adult mosquitoes, crucial differences in vector competence of *Aedes albopictus* for ZIKV depending on the ambient temperature have been demonstrated by Heitmann et al. (2017). German and Italian populations of *Aedes albopictus* that were infected with ZIKV and kept at 18 °C were not found competent for ZIKV transmission (TR of 0%), while a TR of 18–20% was found in *Aedes albopictus* kept at 27 °C after an EIP of 14 days. In contrast, none of the *Culex pipiens* biotype *pipiens*, *Culex pipiens* biotype *molestus*, and *Culex torrentium* populations were found competent at 18 or 27 °C. Similar results were reported for Italian and French *Aedes albopictus* populations (TR of 4–29%) and for an Italian *Culex pipiens* (TR of 0%) population kept at 26 or 28 °C (Boccolini et al. 2016; Di Luca et al. 2016; Jupille et al. 2016). The midgut barriers (MIB and MEB) may be circumvented by using intrathoracic instead of oral infection. Intrathoracically infected mosquitoes show a considerable higher IR and TR (up to 100%) than orally infected mosquitoes as demonstrated for USUV (TR of 69%, *Culex pipiens*) and WNV (TR of 22–33%, *Culex pipiens*) (Fros et al. 2015a, b). This can lead to overestimation of IR and TR and consequently to misleading interpretation of vector competence (Fros et al. 2015a, b). Fu et al. (1999) suggested that, following intrathoracical inoculation, virus levels in the hemocoel exceed the virus amount that can be cleared by fat bodies. Another important factor for efficient infection of mosquitoes is the orally ingested virus dose. Only vertebrate species that produce viremia (sufficiently high for infection) can be regarded as amplifying hosts (Húbalek 2008) as it is the case for WNV in birds, but not for WNV in horses or humans (Angenvoort et al. 2013; Bunning et al. 2002; Hayes et al. 2005). When *Culex quinquefasciatus* mosquitoes are experimentally infected with a low (10^4 plaque-forming units per mL (PFU/mL)) or a higher (10^6 PFU/mL) dose of ZIKV, only mosquitoes that ingested the higher dose got infected (Guedes et al. 2017). In

Table 9.1 Summary of experimental studies of vector competence for ZIKV, DENV, CHIKV, USUV, RVFV, JEV, and WNV of mosquito species collected in Europe (data of positive control mosquito vectors used in the studies are not shown)

Virus		Mosquito		Trial (all o.f.)				Result	Reference
[a]	Origin	Species	Origin/no. field/lab/NA	Temperature (°C)/relative humidity (%)	EIP (days)	Virus detection method	TR (%)/no. of colonies	Vector competent (yes/no)	
CHIKV	La Réunion, strain E1A226V	*Aedes albopictus*	Italy/2f	28/NA	14	PCR	NA (10–100)	Y	Talbalaghi et al. (2010)
CHIKV	La Réunion, strain E1A226V	*Aedes albopictus*	Corsica/2f	28/NA	14	PCR	NA (80–100)	Y	Moutailler et al. (2009)
CHIKV	Strain NC/2011-568	*Aedes detritus*	UK/1f	21/70	17	PCR	0	N	Blagrove et al. (2016)
DENV	Thailand, serotype 2	*Aedes detritus*	UK/1f	21/70	17	PCR	0	N	Blagrove et al. (2016)
JEV	cDNA clones[b]: 1. Taiwan, 1985, g3 strain RP-9 or 2. China, 2009, g5 strain XZ0934	*Aedes albopictus*	France/1l	26/80	13	FFA	20–63%	Y	De Wispelaere et al. (2017)
JEV	Malaysia, 1952, g5, Muar strain	*Aedes detritus*	UK/1f	23/70–90 28/70–90	7–21 14 7–21 14	Vi	13 3 25 17	Y	Mackenzie-Impoinvil et al. (2015)
JEV	Japan, 1935, g3, strain Nakayama[c]	*Aedes japonicus japonicus*	Germany/1f	25/85	14	PCR	100[d]	Y	Huber et al. (2014a)

(continued)

Table 9.1 (continued)

Virus		Mosquito		Trial (all o.f.)				Result	Reference
[a]	Origin	Species	Origin/no. field/lab/NA	Temperature (°C)/relative humidity (%)	EIP (days)	Virus detection method	TR (%)/no. of colonies	Vector competent (yes/no)	
JEV	cDNA clones[b]: 1. Taiwan, 1985, g3 strain RP-9 or 2. China, 2009, g5 strain XZ0934	*Culex pipiens*	France/1l	26/80	13	FFA	12–41%	Y	De Wispelaere et al. (2017)
RVFV	South Africa/strain RVF 56/74	*Aedes albopictus*	Spain/1f	Ø 22 night to Ø 26 day/80	14	Vi	NA (n = 1)	Y	Brustolin et al. (2017)
RVFV	Avirulent Clone 13[e]	*Aedes caspius*	France/1f	28/80	14	IFA	7[d]	Y	Moutailler et al. (2008)
RVFV	Virulent ZH548 avirul. Clone 13[e]	*Aedes detritus*	France/1f	28/80	14	IFA	13[d] 0[d]	Y	Moutailler et al. (2008)
RVFV	Virulent ZH548 avirul. Clone 13[e]	*Aedes vexans*	Germany/1l	28/80	14	IFA	8[d] 25[d]	Y	Moutailler et al. (2008)
RVFV	Virulent ZH548 avirul. Clone 13[e]	*Culex pipiens*	France/1f France/2f	28/80	14	IFA	14[d] 4–9[d]	Y	Moutailler et al. (2008)
RVFV	Virulent ZH548 avirul. Clone 13[e]	*Culex pipiens*	Cyprus/1NA	28/80	14	IFA	30[d] 14[d]	Y	Moutailler et al. (2008)
RVFV	South Africa/strain RVF 56/74	*Cx p.pipiens* b. *molestus*	Spain/1f	Ø 22 night to Ø 26 day/80	14	Vi	0	N	Brustolin et al. (2017)

RVFV	South Africa/strain RVF 56/74	*Cx p.pipiens* b. *pipiens x* b. *molestus* (hybrid)	Spain/1f	Ø 22 night to Ø 26 day/80	14	Vi and/or PCR	NA (n = 6)	Y	Brustolin et al. (2017)
USUV	Italy, 2011, 3 strains[c]	*Aedes albopictus*	Italy/1f	28 ± 1/80	14	PCR	0	N	Puggioli et al. (2017)
USUV	USUV, Bologna/09[c]	*Culex pipiens*	NL/1l	28/60	14	Vi	69%	Y	Fros et al. (2015b)
WNV	France, lineage 1, Camargue 2001, Eva Ref-2651	*Aedes albopictus*	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	NA (1 group)	Y	Brustolin et al. (2016)
WNV	Italy, lineage 2, strain 178907/2013	*Aedes albopictus*	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	NA (1 group)	Y	Brustolin et al. (2016)
WNV	Sardinia, 2011, lineage 1, strain Ma V3	*Aedes albopictus*	Italy/1f	27 ± 1/70	14	Vi	50	Y	Fortuna et al. (2015a)
WNV	USA, lineage 1, strain NY99	*Aedes japonicus japonicus*	Germany/1f	25/85	14	PCR	0[d]	N	Huber et al. (2014a, b)
WNV	USA, lineage 1, strain NY-99, NCBI DQ211652	*Aedes japonicus japonicus*	Switzerland/1f	24 ± 7/45–90	12 to 15	Vi	NA (4 pools)	Y	Wagner et al. (2018)
WNV	Italy, lineage 1, strain Italy/2009/FIN[c]	*Aedes japonicus japonicus*	Switzerland/1f	24 ± 7/45–90	12 to 15	Vi	NA (1 pool)	Y	Wagner et al. (2018)

(continued)

Table 9.1 (continued)

Virus		Mosquito		Trial (all o.f.)				Result	Reference
[a]	Origin	Species	Origin/no. field/lab/NA	Temperature (°C)/relative humidity (%)	EIP (days)	Virus detection method	TR (%)/no. of colonies	Vector competent (yes/no)	
WNV	USA, lineage 1, strain NY-99	*Aedes detritus*	UK/1f	21/70	17	PCR	21	Y	Blagrove et al. (2016)
WNV	USA, lineage 1, strain NY-99[c]	*Culex pipiens*	Switzerland/1f	24 ± 7/45–90	12 to 15	Vi	NA (4 pools)	Y	Wagner et al. (2018)
WNV	Italy, lineage 1, strain Italy/2009/FIN[c]	*Culex pipiens*	Switzerland/1f	24 ± 7/45–90	12 to 15	Vi	0	N	Wagner et al. (2018)
WNV	Sardinia, 2011, lineage 1, strain Ma V3	*Culex pipiens*	Italy/2f, 2l	28 ± 1/70	6 to 32	Vi	37 to 47	Y	Fortuna et al. (2015b)
WNV	Sardinia, 2011, lineage 1, strain Ma V3	*Culex pipiens*	Italy/1f	27 ± 1/70	14	Vi	33	Y	Fortuna et al. (2015a)
WNV	USA, lineage 1, strain NY-99	*Culex pipiens*	NL/1l	23/60	14	Vi	22	Y	Fros et al. (2015a)
WNV	Greece, lineage 2, Gr-2010	*Culex pipiens*	NL/1l	23/60	14	Vi	24	Y	Fros et al. (2015a)
WNV	Greece, lineage 2, Gr-2010[c]	*Culex pipiens*	NL/1l	28/60	14	Vi	33	Y	Fros et al. (2015b)

WNV	France, lineage 1, Camargue 2001, Eva Ref-2651	*Cx p.pipiens* b. *molestus*	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	0	N	Brustolin et al. (2016)
WNV	Italy, lineage 2, strain 178907/2013	*Cx p.pipiens* b. *molestus*	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	0	N	Brustolin et al. (2016)
WNV	France, lineage 1, Camargue 2001, Eva Ref-2651	*Cx p.pipiens* b. *pipiens x b. molestus* (hybrid)	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	0	N	Brustolin et al. (2016)
WNV	Italy, lineage 2, strain 178907/2013	*Cx p.pipiens* b. *pipiens* x *Cx p.pipiens* b. *molestus* (hybrid)	Spain/1f	Ø 21.3 night to Ø 27.7 day/70	12	Vi	NA (1 group)	Y	Brustolin et al. (2016)
ZIKV	Asian genotype?	*Aedes albopictus*	Italy/1NA	18/80 27/80	14 14	Vi Vi	0 18	N Y	Heitmann et al. (2017)
ZIKV	Asian genotype?	*Aedes albopictus*	Germany/1NA	18/80 27/80	14 14	Vi Vi	0 20	N Y	Heitmann et al. (2017)
ZIKV	Asian genotype	*Aedes albopictus*	France/1f	28 ± 1/80	14	Vi	4	Y	Jupille et al. (2016)
ZIKV	Asian genotype	*Aedes albopictus*	Italy/1f	26 ± 1/70	11 to 14	PCR	29	Y	Di Luca et al. (2016)
ZIKV	Asian genotype?	*Cx p.pipiens* b. *pipiens*	Germany/1NA	18/80 27/80	14 14	Vi Vi	0 0	N N	Heitmann et al. (2017)
ZIKV	Asian genotype?	*Cx p.pipiens* b. *molestus*	Germany/1NA	18/80 27/80	14 14	Vi Vi	0 0	N N	Heitmann et al. (2017)

(continued)

Table 9.1 (continued)

Virus		Mosquito		Trial (all o.f.)				Result	Reference
[a]	Origin	Species	Origin/no. field/lab/NA	Temperature (°C)/relative humidity (%)	EIP (days)	Virus detection method	TR (%)/ no. of colonies	Vector competent (yes/no)	
ZIKV	Asian genotype?	*Culex torrentium*	Germany/1NA	18/80 27/80	14 14	Vi Vi	0 0	N N	Heitmann et al. (2017)
ZIKV	Asian genotype	*Culex pipiens*	Italy/1f	26±1/70	14	Vi	0	N	Boccolini et al. (2016)

The transmission rate (TR) is defined as the proportion of mosquitoes with virus-infected saliva or salivary glands (RNA or infectious virus) with respect to the number of mosquitoes with infected body and is considered the most reliable method to investigate the competence of a vector to transmit a virus (except for direct virus transmission to vertebrate hosts by infected mosquitoes). Therefore, and for reasons of clarity, infection and dissemination rates were omitted

b, biotype, *EIP* extrinsic incubation period (days after infection with virus-containing blood meal) is shown for all studies at around an EIP of 14 days to allow comparison of all studies, *FFA* foci-forming assay (quantification by FFU/mL), *g* genotype, *IFA* immunofluorescence assay: evaluate disseminated infection rate (surviving females were tested for the presence of RVFV on head squashes by IFA after an EIP of 14 days), *NA* information not available, *no. field/lab/NA* number of different populations tested collected in the field (f)/obtained from a laboratory colony (l)/unknown (NA), *o.f.* oral feeding (all mosquitoes in the presented studies were fed with a blood meal containing infectious virus), *PCR/Vi* detection of virus genome (RNA) by polymerase chain reaction (PCR) or detection of infectious virus by virus isolation (Vi) assay in saliva or salivary glands, *TR* (%) transmission rate (TR is defined as the number of mosquitoes with virus-positive saliva per number of virus-positive mosquito bodies and (Heitmann et al. 2017) provides the most important information about the vector competence of a mosquito since only virus transmission by saliva during an insect bite is infectious for the vertebrate host is quantified), *NL* The Netherlands, *UK* United Kingdom, Ø mean temperature value

[a]By species, genus, family, order (International Committee on Taxonomy of Viruses (ICTV), https://talk.ictvonline.org/taxonomy, 2017): CHIKV, *Chikungunya virus*, *Alphavirus*, *Togaviridae*; DENV, *Dengue virus*, *Flavivirus*, *Flaviviridae*; JEV, *Japanese encephalitis virus*, *Flavivirus*, *Flaviviridae*; RVFV, *Rift Valley fever phlebovirus*, *Phlebovirus*, *Phenuivirus*, *Phenuiviridae*, *Bunyavirales*; USUV, *Usutu virus*, *Flavivirus*, *Flaviviridae;* WNV, *West Nile virus*, *Flavivirus*, *Flaviviridae*; ZIKV, *Zika virus*, *Flavivirus*, *Flaviviridae*

[b]Molecular cDNA clones of JEV genotype 3 strain RP-9 and JEV genotype 5 strain XZ0934 (de Wispelaere et al. 2017) were transfected in HEK293T cells and grown in DF-1 cells

[c]NCBI accession numbers (USUV1, KF055442; USUV2, KF055441; USUV3, KF055440; Italy/2009/FIN, KF234080 (lineage 1 according to Lim et al. 2013)); NY-99, DQ211652; USUV Bologna/09, HM569263; WNV Gr-2010 lineage, HQ537483.1; JEV genotype 3 strain Nakayama, EF571853

[d]Virus RNA detection (Huber et al. 2014a, b) or virus staining by IFA (Moutailler et al. 2008) in whole head samples (not directly in saliva or salivary glands)

[e]Virulent RVFV strain ZH548, Egypt & avirulent Clone 13, Central African Republic, Bangui

contrast, a considerably higher TR was found in *Aedes vexans* originating from a German colony infected with an avirulent RVFV strain (Clone 13) (TR of 25%) compared to a virulent RVFV strain (ZH548) (TR of 8.3%) (Moutailler et al. 2008).

In European mosquito populations, transovarial transmission has only been investigated by Fortuna et al. (2015b) in four different *Culex pipiens* populations collected in Italy and experimentally infected with WNV. However, vertical transmission could not be confirmed in their offspring, although all four populations showed similar TR in their saliva (TR of 37–47%) and were therefore vector competent (Fortuna et al. 2015a) (Table 9.1). In contrast, transovarial transmission was found for WNV in *Culex vishnui* in India (Mishra and Mourya 2001) and for an insect-specific flavivirus (*Culex flavivirus*) by American *Culex pipiens* (Saiyasombat et al. 2011). *Bagaza virus* (*Flaviviridae)* was transovarially transmitted by *Culex tritaeniorhynchus* from India, but not by *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes (Sudeep et al. 2013). Various studies in non-European countries confirmed the possibility of natural transovarial transmission by *Aedes aegypti* for different viruses such as DENV and ZIKV by analysis of immature mosquito stages (Gutiérrez-Bugallo et al. 2017; Li et al. 2017; Velandia-Romero et al. 2017). A high percentage of transovarial transmission of DENV (54.7% of immature stages in households) together with the possibility of transmission by the vector without a prior blood meal has been suggested a possible explanation for the persistence of DENV in (rural) areas (Velandia-Romero et al. 2017). However, the impact of transovarial transmission for DENV in other regions was found negligible, scrutinizing the elimination of larvae as intervention methods (Angel et al. 2016). On the other hand, elimination of larvae is not considered a powerful method for vector control (Pfeffer 2015) (see section vectorial capacity).

9.2.3 Vectorial Capacity

The vectorial capacity (VC) is defined as the efficiency of a mosquito species to serve as a vector for a given pathogen and can be estimated using calculations of the basic reproductive rate (R_0). VC is an entomological restatement of R_0 of a pathogen (Kramer 2016; Schaffner and Mathis 2014). R_0 is defined as the number of secondary infections expected to occur from the introduction of a single infection in a naïve population (Kramer 2016), and a key method to understand disease transmission. A major epizootic outbreak and spread of disease within a population are expected if $R_0 > 1$, while minor disease outbreaks that become extinct are expected if $R_0 < 1$. R_0 can be used to plan strategies for control of epizootics but also to estimate, quantify, and compare the outcome of control measures (Pfeffer 2015; Weesendorp et al. 2011). Out of different published equations, the following was proposed by Kramer (2016) and Pfeffer (2015):

$$R_0 = \mathrm{VC} = ma^2 \left(\mathrm{IR}^{*}\mathrm{TR}\right) p^t \,/ -\ln\left(p\right)$$

VC, vectorial capacity (R_0)

m, vector density in relation to the vertebrate host

a, probability that vector feeds on a host in 1 day (i.e., host preference index * feeding frequency)

p, probability that vector survives one day

t, duration of extrinsic incubation period (EIP) in days (latency period)

IR, infection rate (proportion of vectors infected after feeding on an viremic host)

TR, transmission rate (proportion of infected vectors that are able to transmit the virus to a host)

(IR * TR), vector competence (proportion of vectors ingesting an infective blood meal that are later able to transmit the infection to a host)

1/−ln (*p*), duration of the vector's life in days after surviving the EIP (recovery rate)

Accordingly, viral factors are of major importance: a rapid dissemination of a virus from the midgut to the salivary glands would reduce the EIP and, hence, at the same time prolong the duration of the vector's life after surviving the EIP (=1/−ln (*p*)). In contrast, host feeding (*a*), vector longevity (*p*), and EIP (*t*) would have a more powerful impact on VC (as square or component), while the vector-to-host density relation (*m*) and vector competence (IR * TR) of a mosquito population would have a linear and therefore weak effect on VC (Kramer 2016).

The control of malaria (caused by parasitic *Plasmodium* spp.) is a vivid example to demonstrate the power or weakness of different control strategies. Control of mosquito larvae affects the vector-host proportion, but a reduction of larvae (*m*) by 50% only results in a 50% reduction of the VC. However, a reduction of the daily survival time of mosquito vectors of *Plasmodium* (*p*) by 50% results in a 1000 times lower proportion of mosquitoes that transmit malaria since a reduction of *p* (survival time) has a direct effect on EIP (*t*) and the recovery rate (1/−ln (*p*)) (Pfeffer 2015).

9.2.4 Outcome of Experimental Vector Competence Studies by Virus Species

9.2.4.1 CHIKV

Aedes albopictus, one of the most invasive mosquitoes now endemic across southern Europe, was the main vector for the initial CHIKV outbreak in Italy in 2007 (Bonilauri et al. 2008). *Aedes aegypti*, another primary vector of CHIKV, was introduced in Madeira (Portugal) in 2005 (CDC 2017; Sigfrid et al. 2017). Further autochthonous chikungunya outbreaks were reported in France in 2010 and 2014 (Delisle et al. 2015; Gould et al. 2010). The risk of CHIKV introduction and spread in Europe are highlighted by recent autochthonous outbreak of chikungunya in Italy and spread to France in 2017 (CDC 2017). Bioassays for vector competence studies have been conducted with four different *Aedes albopictus* field populations

collected in Italy (n = 2) and Corsica, France (n = 2) (Moutailler et al. 2009; Talbalaghi et al. 2010). In both experiments, mosquitoes were infected with a CHIKV strain from the island La Réunion and kept at 28 °C. The TR was approximately between 10 and 80% up to 100% (Moutailler et al. 2009; Talbalaghi et al. 2010). These results are similar to the TR (61%) measured in a US *Aedes aegypti* strain infected with another CHIKV isolate (Blagrove et al. 2016). However, the latter experiment was conducted at a considerably lower temperature (21 °C). In comparison to the main vectors of CHIKV, the mosquito species *Aedes detritus* endemic to the UK, and a possible vector of JEV, RVFV, and WNV (Table 9.1), was CHIKV-infected and kept under the same experimental settings as *Aedes aegypti* (Blagrove et al. 2016). In contrast to *Aedes aegypti*, *Aedes detritus* was not susceptible to CHIKV infection, at least in this experimental setting (Blagrove et al. 2016). However, higher temperatures during the infection experiment or during the maturation of insects may affect their vector competence (Kramer 2016; Lourenço-de-Oliveira et al. 2013; Mellor 2009) for CHIKV. Hence, further vector competence studies are needed for abundant European mosquito species such as *Culex pipiens* and *Aedes vexans* to analyze their vector competence for CHIKV. A study of dissemination rates (DR) in Italian populations of *Anopheles maculipennis* (0%), *Aedes vexans* (7.7%), and *Culex pipiens* (0–33%) after CHIKV infection showed low susceptibilities suggesting a negligible role of these European mosquito species for CHIKV transmission (Talbalaghi et al. 2010).

9.2.4.2 DENV

A large increase in dengue fever cases has been experienced around the globe in the past decades. Between 2010 and 2014, repeated sporadic or large outbreaks have been reported in over 20 European countries (Kampen and Werner 2015; Sigfrid et al. 2017; WHO 2017). *Aedes aegypti* and *Aedes albopictus* are considered the two main vectors of DENV. Infection by one of the four DENV serotypes (DENV-1 to DENV-4) only mediates partial and temporary cross-immunity. Even more, additional infections with other serotypes can lead to severe dengue (Dejnirattisai et al. 2010). Despite to permanent risk of DENV introduction to Europe, only a few studies on the vector competence of European mosquito species have been conducted. One study uses British *Aedes detritus* and tropical *Aedes aegypti* mosquitoes for DENV infection and kept the mosquitoes at a low ambient temperature of 21 °C and 70% RH after infection to simulate low temperate temperatures of Great Britain (Blagrove et al. 2016). Similar to the results of CHIKV infection in these mosquito strains, *Aedes detritus* was not susceptible to DENV-2, while *Aedes aegypti* showed a high TR of 70%. Talbalaghi et al. (2010) and Moutailler et al. (2009) investigated dissemination rates (DR) of Italian and Corsican (France) *Aedes albopictus* populations after infection with DENV-2, but not TR. Italian *Aedes albopictus* (14–39%) and Corsican *Aedes albopictus* (13–69%) kept at 28 °C for 14 days showed similar DR. Because of intrinsic barriers in the mosquito potentially interfering with transmission, the TR as a proxy for vector competence is not necessarily similar to

DR. Thus, vector competence for European mosquito populations of *Aedes albopictus* is not confirmed yet, but is likely considering the global role of *Aedes albopictus* as vector of DENV. Further studies are needed to investigate the vector competence for various potential European mosquito vectors and the four DENV serotypes.

9.2.4.3 JEV

JEV is an exotic flavivirus to Europe. However, recent detection of fragmented JEV-RNA in Italian *Culex pipiens* mosquitoes and birds caught in 2010 indicated a sporadic introduction of JEV to Europe, although complementary studies to confirm the presence of JEV in Europe are required (Platonov et al. 2012; Ravanini et al. 2012; Zeller 2012). Several groups therefore aimed to investigate the vector competence of mosquito species endemic (*Aedes detritus* and *Culex pipiens*) or invasive (*Aedes albopictus*, *Aedes japonicus japonicus*) to Europe. While *Aedes albopictus* by now commonly occurs in large parts of Europe (in particular in Southern Europe), *Aedes japonicus japonicus* occurs considerably less frequent in Europe. However, this mosquito species is adapted to temperate regions, has been established in a few regions of Germany since 2008 (Kampen and Werner 2015), and was shown competent for JEV replication (Huber et al. 2014a). All four European mosquito species—*Aedes detritus* collected in the UK, *Culex pipiens* and *Aedes japonicus japonicus* collected in Germany, as well as *Aedes albopictus* collected in France that were orally infected with JEV strains of genotype 3 or 5 (Table 9.1)—were found competent for JEV transmission (de Wispelaere et al. 2017; Huber et al. 2014a; Mackenzie-Impoinvil et al. 2015). De Wispelaere et al. (2017) used two cDNA clones of field strains after their rescue in cell culture, while all other groups used field strains. *Aedes albopictus*, *Aedes japonicus japonicus*, and *Culex pipiens* species were kept at 25 or 26 °C and 80–85% RH, simulating intermediate to diurnal summer temperatures of Mediterranean Europe. TR ranged between 12 and 63% for *Aedes albopictus* and *Culex pipiens*. For *Aedes japonicus japonicus*, only the DR in the whole head (analyzed by PCR) was investigated, which was considerably higher (100%) (Huber et al. 2014a) compared to the TR of JEV found for the other mosquito species. Therefore, the high DR cannot necessarily be used to draw conclusions for the TR, which requires analyses of saliva or at least salivary glands (see section barriers). The study was included in this review since no other studies of vector competence for JEV in European *Aedes japonicus japonicus* mosquito populations have been conducted so far. The vector competence of local (temperate) British *Aedes detritus* mosquitoes was comparatively analyzed using 23 or 28 °C and a RH range of 70–90%. Interestingly, *Aedes detritus* mosquitoes were found competent at both temperatures, although the RT was markedly lower at 23 °C (TR of 3%) compared to 28 °C (TR of 17%). Interestingly, similar TRs were obtained for *Culex quinquefasciatus*, a tropical mosquito previously incriminated as vector for JEV (Mackenzie-Impoinvil et al. 2015). In summary, the results of the vector competence studies of JEV in three commonly occurring mosquito species in

Europe suggest that JEV transmission is possible in various European countries especially during warm summer nights and in Mediterranean Europe. Complementary studies are necessary to determine the vector competence of different *Aedes japonicus japonicus* populations invasive in Europe for JEV. The results of the vector competence studies together with the recent detection of fragmented RNA of a JEV or a related virus highlight the need for comprehensive surveys of JEV in different mosquito species in Europe.

9.2.4.4 RVFV

RVFV is an arbovirus mainly transmitted by a large number of different mosquito species to different mammals including humans in Africa. Multiple outbreaks of RVFV outside Africa, particularly in countries bordering the Mediterranean Sea, point to a high probability of RVFV outbreaks in Europe. Key drivers of seasonally high numbers of RVF disease outbreaks are heavy rainfalls following periods of drought that suddenly increase vector density (due to rain associated hatching of larvae to imago). The high vector density at water holes leads to a high probability of infection of susceptible vertebrate hosts that regularly visit water holes for drinking. The possibility of transovarial transmission of RVFV to the mosquito offspring as reported by Linthicum et al. (1985) contributes to efficient transmission of this virus (Brustolin et al. 2017; Moutailler et al. 2008).

Vector competence studies for RVFV in European mosquito species are scarce. Oral infection of Spanish *Aedes albopictus*, *Culex pipiens* biotype *molestus*, and hybrid *Culex pipiens* biotype *pipiens* x *molestus* with an South African RVFV strain resulted in the release of infectious virus transmission in saliva of a few individuals belonging to the species *Aedes albopictus* and the hybrid *Culex pipiens* biotype *pipiens* x *molestus* (exact proportion of the TR was not given) but not of the species *Culex pipiens* biotype *molestus* (Brustolin et al. 2017). The midgut barriers of infection (MIB) and escape (MEB) were comparatively analyzed in the species *Culex pipiens* biotype *molestus* and the hybrid species by virus isolation. Two different viral doses were used for oral infection (5.7 $\log_{10}TCID_{50}$/mL or 5.7 $\log_{10}TCID_{50}$/mL). Interestingly, while the lower and higher doses resulted in infection of the MIB in both species (IR of 7–20%), the MEB was only overcome in hybrid *Culex pipiens* biotype *pipiens* x *molestus* after infection with the higher virus dose (DR of 66.6%), but not in the species *Culex pipiens* biotype *molestus* (0%). A similar dependence of the viral dose on the infection and escape of midgut cells was previously reported for BTV in *Culicoides* (Mellor 2009). On the other hand, *Culex pipiens* biotype *molestus* is generally refractory to infection with various other viruses (WNV lineages 1 and 2, and ZIKV) (Brustolin et al. 2017; Heitmann et al. 2017) (Tables 9.1 and 9.2). Moutailler et al. (2008) studied various European mosquito species regarding their vector potential for RVFV by analyzing virus in head squashes by immunofluorescence assay, and hence the DR but not TR. At 14 days postinfection, *Aedes vexans* showed a considerably lower DR in virulent RVFV (ZH548, 8.3%) compared to an avirulent strain (Clone13, 25%) (Moutailler

Table 9.2 Vector competence of different mosquito-vector species for endemic and emerging pathogens in Europe

Mosquito species	Experimentally confirmed vector competence[a]	Experimentally confirmed lack of vector competence[a]	Collective field and experimental results[b]
Aedes albopictus	SINV[c], CHIKV, JEV, RVFV, WNV L1, WNV L2, ZIKV	USUV	CHIKV, DENV
Aedes caspius	RVFV	–	WNV, SINV, TAHV, USUV
Aedes detritus	JEV, RVFV, WNV L1	CHIKV, DENV	USUV
Aedes japonicus japonicus	JEV, WNV L1	WNV L1	WNV, SINV, TAHV, USUV, RVFV
Aedes vexans	RVFV	–	WNV, SINV, TAHV, USUV, RVFV
Culex pipiens	JEV, RVFV, USUV, WNV L1	WNV L2, ZIKV	WNV, SINV, TAHV, USUV, RVFV
Culex p.p. b. *molestus*	–	RVFV, WNV L1, WNV L2, ZIKV	–
Culex p.p. b. *pipiens*	–	ZIKV	–
Culex p.p. b. *pipiens* x b. *molestus* (hybrid)	RVFV, WNV L2	WNV L1, ZIKV	–
Culex torrentium	–	ZIKV	SINV

[a]Summary of vector competence studies by mosquito species (as described in Table 9.1), and, for comparison, [b]collective results of European field studies and experimental studies as reviewed by Kampen and Werner (2015), Húbalek (2008), and Nikolay (2015)
L lineage, *p. pipiens*, b. biotype, - no information available
SINV, *Sindbis virus* (*Alphavirus, Togaviridae*); TAHV, *Tahyna virus*, infraspecies of *California encephalitis virus, Peribunyaviridae*); [c]result of experimental infection of *Aedes albopictus* with SINV by Dohm et al (1995); references for [a] according to Table 9.1: CHIKV, *Chikungunya virus* (Talbalaghi et al. 2010; Moutailler et al. 2009; Blagrove et al. 2016); DENV, *Dengue virus* (Blagrove et al. 2016); JEV, *Japanese encephalitis virus* (Huber et al. 2014a, b; Mackenzie-Impoinvil et al. 2015; de Wispelaere et al. 2017); RVFV, *Rift Valley fever phlebovirus* (Brustolin et al. 2017; Moutailler et al. 2008); USUV, *Usutu virus* (Puggioli et al. 2017; Fros et al. 2015b); WNV, *West Nile virus* (WNV L1: Brustolin et al. 2016; Fortuna et al. 2015a; Fortuna et al. 2015b; Huber et al. 2014a, b; Wagner et al. 2018; Blagrove et al. 2016; Fros et al. 2015a; WNV L2: Brustolin et al. 2016; Fros et al. 2015a; Fros et al. 2015b); ZIKV, *Zika virus* (Heitmann et al. 2017; Jupille et al. 2016; Di Luca et al. 2016; Boccolini et al. 2016); Werner et al. 2015; Húbalek 2008)

et al. 2008). In contrast, for the three European mosquito species, namely, *Aedes detritus*, *Culex pipiens* (France), and *Culex pipiens* (Cyprus) infected with both RVFV strains, DR were markedly higher after infection with the virulent ZH548 (13–30%) compared to the avirulent Clone 13 strain (0–14%) (Table 9.1). In the

French colonies of *Aedes caspius* (7%) and *Culex pipiens* (9%) infected with the avirulent Clone 13 RVFV strain, DR were similarly low (7 and 9%, respectively) (Moutailler et al. 2008). In addition to vector competence of the European mosquitoes, results were compared with field strains of different *Aedes* and *Culex* species from different African and Asian countries. In general, similar dissemination of the virus is found in all tested species compared with the DR results of the European mosquito species, except for *Aedes aegypti*. *Aedes aegypti* showed a considerably higher DR of 20–90% for the virulent RVFV ZH458 and 24–73% for the avirulent Clone 13 strain suggesting that transmission of RVFV by *Aedes aegypti* is more efficient (Moutailler et al. 2008). On the other hand, mosquitoes belonging to the *Culex pipiens* complex are considered efficient vectors of RVFV in Africa, and virus isolation of RVFV from at least 40 mosquito species (Moutailler et al. 2008) indicates that the broad variety of competent vectors of RVFV primarily contributes to the efficient transmission of this virus in highly diverse habitats and climatic regions. The demonstration of vector competence of Spanish field populations of *Culex pipiens* and *Aedes albopictus* for RVFV and the potential vector competence of other European mosquitoes indicate that autochthonous outbreaks of RVFV are possible in Southern Europe.

9.2.4.5 Usutu Virus

In a comprehensive field study of USUV infection in different mosquito species in Italy from 2009 to 2012, a substantial incidence of *Aedes albopictus* mosquitoes PCR-positive for USUV was found. However, USUV was not detected in any of the *Aedes albopictus* specimens collected in 2013 (Puggioli et al. 2017). Experimental infection of *Aedes albopictus* collected in the field in Italy with any of the three Italian virus strains (of 2011) and incubation at 28 °C and 80% RH showed RNA in a single individual after an EIP of 7 days, but no mosquitoes were found PCR-positive after an EIP of 14 days. Therefore, Puggioli et al. (2017) suggested that *Aedes albopictus* plays a negligible role in the epidemiology of USUV, but further studies are necessary using different experimental parameters. In contrast, *Culex pipiens* orally infected with USUV strain Bologna/09 showed a high vector competence (TR of 69%) at an EIP of 14 days at 28 °C and 60% RH, which is significantly higher compared to TR found for *Culex pipiens* infected with WNV lineage 2 strain Gr-2010 (TR of 33%) by the same group (Fros et al. 2015b). A considerable dependence on temperature was found comparing infection rates of *Culex pipiens* mosquitoes kept at 60% RH and the three different temperatures 18 °C (TR of 11%), 23 °C (TR of 53%), and 28 °C (TR of 90%). Since these three different temperatures represent the mean diurnal summer (July–August) temperature in North-Western Europe, an intermediate temperature, and the mean diurnal summer temperature for Mediterranean Europe, respectively, it can be assumed that particularly in Southern Europe, the transmission rate of USUV by *Culex pipiens* is considerably higher (Fros et al. 2015b). In a comprehensive field study of USUV occurrence in different

mosquito species in Germany, USUV was detected or isolated from *Culex pipiens* (Jost et al. 2011a; Sieg et al. 2017). In field studies in Italy (Calzolari et al. 2012; Mancini et al. 2017) and other countries (reviewed in Nikolay (2015)), additional mosquito species were found PCR-positive for USUV, including *Culex pipiens s.l.*, *Aedes albopictus*, *Aedes caspius*, *Aedes detritus*, *Anopheles maculipennis*, and *Culiseta (Cs.) annulata*. Similar to the results of the German studies (Jost et al. 2011a; Sieg et al. 2017), the cumulative results of the Italian field studies confirm that *Culex pipiens* likely is most involved in USUV circulation in Italy (Calzolari et al. 2012; Mancini et al. 2017) and in other European countries.

9.2.4.6 West Nile Virus

In Europe, *Culex pipiens* is considered the main vector of WNV, but other species such as *Aedes albopictus* (Fortuna et al. 2015a), *Aedes detritus*, or *Aedes japonicus japonicus* (Wagner et al. 2018) may also act as competent vectors. Therefore, several research groups investigated the vector competence of these mosquito species in comparison to the main European vector *Culex pipiens* for WNV lineage 1 and 2 strains by using field and laboratory mosquito colonies collected in different European countries. Huber et al. (2014a) did not find replication of North American WNV lineage 1 strain NY-99 in a German *Aedes japonicus japonicus* population after artificial infection, while Wagner et al. (2018) found the *Aedes japonicus japonicus* populations collected in the neighboring country Switzerland susceptible for the same WNV strain and the Italian strain Italy/2009/FIN. *Aedes detritus*, a mosquito species endemic in the UK, were kept at 21 °C and 70% RH (according to climatic conditions in the UK during warmer seasons) during the experiment and were found competent for WNV strain NY-99 infection under these conditions (Blagrove et al. 2016). As expected, *Culex pipiens* endemic in Switzerland were found competent for the replication of WNV strain NY-99 (Wagner et al. 2018). A comparison of vector competence for European WNV lineages 1 and 2 strains was conducted by Brustolin et al. (2016) and Fros et al. (2015b). In contrast to other studies, Brustolin et al. (2016) used a fluctuating temperature regimen (mean of 21.3 °C at night and mean of 27.7 °C during the day, at 70% RH) to mimic natural conditions. For the comparative study of WNV line 1 and 2 strains, *Aedes albopictus*, *Culex pipiens pipiens* biotype *molestus*, and *Culex pipiens pipiens* hybrids of biotypes *pipiens* and *molestus* were collected in the field in Spain and orally infected with European WNV lineage 1 (France 2001) or 2 (Italy 178907/2013). The *Culex pipiens* hybrid was competent for lineage 2 but refractory to WNV lineage 1 (Brustolin et al. 2016). In contrast, *Aedes albopictus* was found competent for both strains (Brustolin et al. 2016). Similarly, a field colony of *Aedes albopictus* collected in Italy and orally infected with the European Sardinia 2011 lineage 1 strain Ma V3 kept at 27 °C and 70% RH showed a high vector competence (TR of 50%) (Fortuna et al. 2015b). A possible reason for a broader vector competence, more efficient transmission of arboviruses, and outbreak establishment might be

that *Aedes albopictus* has a higher genetic variability due to independent and transcontinental introductions (Manni et al. 2017), which could therefore facilitate the adaption of this mosquito species to different regions and climates. Considerable genomic variations in *Aedes japonicus japonicus* due to similar reasons were also suggested by Kampen and Walther (Kampen and Werner 2014; Zielke et al. 2014, 2015, 2016). Fros et al. conducted vector competence studies with a laboratory colony of *Culex pipiens* collected in the Netherlands. After infection with the WNV lineage 1 strain NY-99 and the European lineage 2 strain Gr-2010 and maintenance at 23 °C (mean average temperature in Central Europe) and 28 °C (Mediterranean mean diurnal summer), a similar vector competence for both lineages and a slightly higher transmission rate at a higher temperature (TR of 33% compared to 24%) were found (Fros et al. 2015a, b). Interestingly, the vector competence and dissemination rate of these North-West European *Culex pipiens* was similarly high for both the NY-99 and Gr-2010 strains at 23 °C, while mosquitoes of North American origin infected with the same strains showed a significantly lower transmission rate for the WNV lineage 2 strain (Fros et al. 2015a). Unfortunately, the biotype of the *Culex pipiens* was not described to evaluate whether these mosquitoes were hybrids that may inherit a higher vector competence compared to *Culex pipiens* biotype *molestus* as described by Brustolin (Brustolin et al. 2016).

9.2.4.7 Zika Virus

ZIKV has been circulating in Africa and South-East Asia for over 65 years. However, during the recent ZIKV endemic in the Americas, this Asian ZIKV genotype has been linked to different phenotypic characteristics (including congenital malformation and neurological disorders in humans, higher infection rates in *Aedes aegypti*) compared to the African ZIKV genotype (Willard et al. 2017). A risk analysis of Gardner et al. (2017) revealed that the vector status of *Aedes* species determines geographical risk of autochthonous ZIKV establishment. While the risk is geographically limited if *Aedes aegypti* is the only competent ZIKV vector, vector competence of *Aedes albopictus* would pose a risk of local establishment in all American regions including Canada and Chile, much of Western Europe, Australia, New Zealand, and South and East Asia, with a substantially increase in the risk of ZIKV outbreaks in Asia (Gardner et al. 2017). To estimate the risk of different mosquito species in different climatic regions, European *Aedes albopictus* were collected from the field in Italy, France, and Germany and experimentally infected with ZIKV belonging to the Asian genotype. *Aedes albopictus* were found competent at temperatures between 26 and 28 °C, but refractory to ZIKV at 18 °C (Di Luca et al. 2016; Heitmann et al. 2017; Jupille et al. 2016). In contrast, *Culex pipiens* collected in Italy and kept at 26 °C and 70% RH (Boccolini et al. 2016) as well as *Culex pipiens* biotype *molestus* and biotype *pipiens* and *Culex torrentium* collected in Germany incubated at 18 or 27 °C and 80% RH (Heitmann et al. 2017) were not found competent vectors of the Asian ZIKV genotype.

9.2.5 *Lessons Learned by Experimental Vector Competence Studies*

In summary, the varying results of the research groups regarding the proportion of mosquitoes of the same species that were found competent for WNV transmission (Table 9.1) may be due to considerable variations in specific mosquito genotype and virus genotype interactions (Lambrechts 2010; Lambrechts et al. 2009a). A considerable genetic variability in *Aedes albopictus* and *Aedes japonicus japonicus* due to independent and transcontinental introductions (Kampen and Werner 2015; Manni et al. 2017) can result in a broader vector competence, more efficient transmission of arboviruses, and regional outbreaks. A meta-analysis of laboratory experiments with DENV indicated that colonization of *Aedes albopictus* over a few generations might result in an increase of their susceptibility to DENV infection (Lambrechts 2010).

On the other hand, the effect of virus genotypes, serotypes, or lineages may be underestimated or overestimated regarding virulence and transmissibility for different mosquito populations of the same species. Vertebrate host factors such as differences in resistance to infection or low viremia may considerably impact virus transmission between hosts (Húbalek 2008; Reisen and Hahn 2007). Adaptation of new viruses to local hosts and vectors by initial positive (diversifying) selection with more virulent quasispecies, followed by negative (stabilizing) selection driven by strong evolutionary constraints, is reported for BTV (Boyle et al. 2012, 2014; Maclachlan et al. 2009; Schulz et al. 2016). For example, *Culex pipiens* populations occurring in North America showed a significantly lower transmission rate for a WNV lineage 2 strain compared to North-Western European *Culex pipiens* species, while a similar transmission rate was found for WNV lineage 1 (Fros et al. 2015a). However, even specific combinations of isofemale families and viral isolates may affect quantity of dissemination within mosquito vectors (Lambrechts et al. 2009a), challenging the validity and relevance of laboratory experiments with single virus-mosquito combinations (Lambrechts et al. 2009a). Furthermore, differences in mortality rates of virus-infected mosquitoes might be due to virus factors (see section virus adaptation to mosquitos). *Aedes albopictus* infected with CHIKV died a few days earlier than non-infected mosquitoes, while the primary vector *Aedes aegypti* survived the infection due to antiviral immune response (see section immune response against arboviruses). A higher frequency of cytopathological changes in salivary glands has been reported in WNV-infected mosquitoes (Girard et al. 2007). Furthermore, fast virus dissemination from the midgut impacting the duration of EIP, low mortality rate, and differences in feeding behavior influence the vectorial capacity of a vector (see section vectorial capacity). Interestingly, *Aedes aegypti* infected with DENV showed a significantly prolonged probing time and enhanced feeding frequency (Platt et al. 1997).

Therefore, the vector competence of various vector genotype and virus genotype combinations by studying different populations over time and space (from different regions/countries of interest) may result in an average and collective experience to

allow an estimation of vectorial capacity of a mosquito species from different areas and over time (Fonseca 2016). In addition, a bias in results of vectorial capacity due to variations in methodologies used by different research groups may be mitigated by analyses of similar virus genotype and mosquito genotype combinations (Lambrechts 2010; Lambrechts et al. 2009a). On the other hand, harmonization of methods (e.g., temperature regimes) and analyses of experiments (representation of proportions of transmission rates by species) as well as the meticulous description of the origin and taxonomy of the used mosquito vectors and virus strains would be most valuable in terms of comparability and reproducibility. In a considerable number of studies, *Culex pipiens* was only superficially taxonomically classified. However, comparison of results of experimental infection of the *Culex pipiens* biotype *pipiens*, *Culex pipiens* biotype *molestus*, and hybrids of both forms revealed considerable differences in their susceptibility to different virus species and lineages (RVFV, WNV lineages 1 and 2, and ZIKV) under equal or similar experimental conditions (Brustolin et al. 2016, 2017; Heitmann et al. 2017) (Table 9.1) insofar that the parental forms *molestus* and *pipiens* of *Culex pipiens* seem to be refractory to the so far tested viruses (Table 9.2), while hybrids of *Culex pipiens* biotype *pipiens* and *molestus* were found competent for RVFV and WNV lineage 2 (Tables 9.1 and 9.2).

Change in climate, land use, genetic diversity within mosquito species in combination with a rapid arboviral adaptation to alternative mosquito, and vertebrate hosts constitute a dynamic system that can substantially and rapidly change the epidemiological patterns of a viral disease as well as the disease expression in vertebrate hosts and therefore the impact on animal welfare and economy of affected countries (Kramer 2016; Lambrechts 2010; Schulz et al. 2016).

9.3 How Do Viruses and Vectors Interact to Facilitate Transmission?

Arboviruses can efficiently replicate in evolutionary distinct hosts, such as mosquitoes and humans; yet they seem to depend on specific mosquito vectors for transmission. The intrinsic factors that determine whether a specific mosquito can transmit a given virus (vector competence) remain poorly understood. Major factors defining vector competence of mosquito species are (1) the control of viral replication by the mosquito to an extent that the mosquito itself is not affected by the virus, (2) virus adaptation to the mosquito to increase viral replication, and (3) the microbiome in the insect vector (illustrated in Fig. 9.3). Within this part, a brief overview of these factors will be given, but since these factors are subject to intense research these days, not all details can be given in the frame of this chapter. For more detailed information please refer to recent reviews (Blair and Olson 2015; Donald et al. 2012; Johnson 2015; Sim et al. 2014).

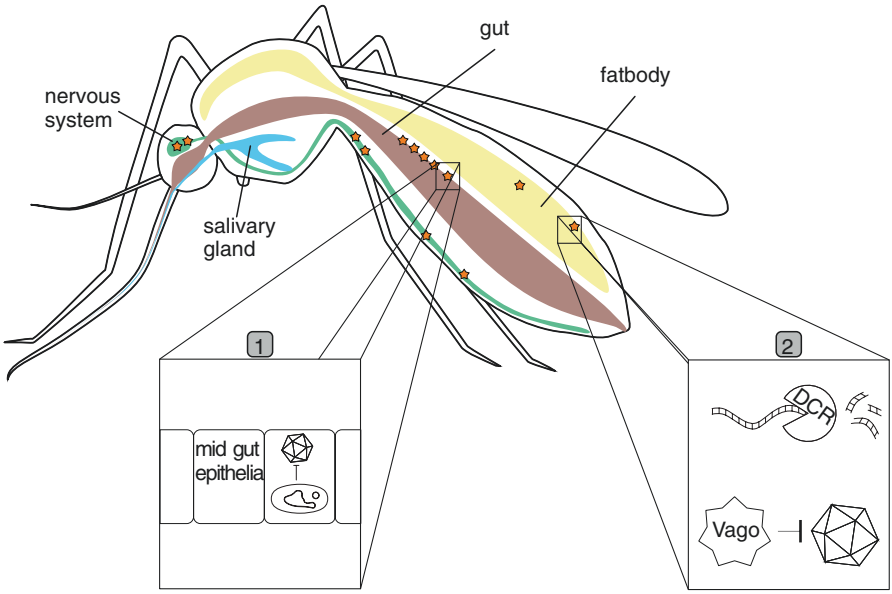

Fig. 9.3 Intrinsic factors that interfere with the vector competence of mosquitoes. The vector competence of a certain mosquito species is characterized by several factors: firstly, the ability of the virus to overcome the midgut barrier (midgut infection and escape barrier), secondly the ability of the virus to replicate in various tissues of the insect host, and most importantly the efficient dissemination of infectious viral particles to the saliva (salivary gland infection and escape barrier). The virus replication in the mosquito midgut is regulated by the gut microbiota represented by the *Wolbachia* endosymbiont (1) which can interfere with the virus replication by various ways including immune priming and completion for resources. After the dissemination of the virus to different mosquito organs such as the fat body and endothelial cells, the virus starts to replicate in these different tissues. The virus replication can trigger several antiviral pathways such as the RNAi pathways represented by Dicer cleavage and the inducible immune responses represented by the induction of *Vago* (2)

9.3.1 Immune Response in Insects Against Arboviruses

9.3.1.1 RNAi Responses

Lacking an adaptive immune system, insects depend on different immune mechanisms for antiviral defense. Using the model insect *Drosophila melanogaster*, it has been demonstrated that RNA interference (RNAi) pathways are crucial to control various *Drosophila* viruses and also metazoonotic viruses such as SINV, WNV, *v Vesicular stomatitis virus* (VSV), and DENV (Chotkowski et al. 2008; Galiana-Arnoux et al. 2006; Mukherjee and Hanley 2010; van Rij et al. 2006; Wang et al. 2006; Zambon et al. 2006). The exogenous (antiviral) siRNA pathway (exoRNAi) is initiated by recognition and cleavage of long double-stranded (ds) RNA, deriving from viral replication intermediates or secondary RNA structures in viral genomes, by the RNaseIII enzyme Dicer-2 (Dcr-2). The resulting 21 nucleotide (nt)-long

virus-derived small interfering RNAs (viRNAs) are then subjected to a multiprotein RNA-induced silencing complex (RISC). In this complex, the major component Argonaute-2 (Ago2) together with one strand of the viRNA initiates the sequence-specific degradation of viral genomes or transcription products (Liu et al. 2006; van Rij et al. 2006). Survival experiments in *Drosophila* lacking the key components of a functional exoRNAi response have demonstrated the exoRNAi-mediated control of arbovirus replication is crucial for the insects' survival (Dietrich et al. 2017a; Kemp et al. 2013; Mueller et al. 2010; Mukherjee and Hanley 2010). The sequencing of full genomes of *Aedes aegypti* (Nene et al. 2007), *Culex quinquefasciatus* (Arensburger et al. 2010), and *Anopheles gambiae* (Holt et al. 2002) enabled the identification of orthologues of *Dcr-2* and *Ago2* in three important vector mosquito species (Campbell et al. 2008a) and subsequent description of further *Dcr-2* and *Ago2* orthologues in more vector species such as *Aedes albopictus* (Brackney et al. 2010). Furthermore, the production of viRNAs, a hallmark of exoRNAi pathway induction, has been shown in *Aedes* and *Culex* mosquitoes response to infection of mosquitoes with different arboviruses (Blair and Olson 2015; Brackney et al. 2010; Campbell et al. 2008b; Carissimo et al. 2015; Dietrich et al. 2017a, b; Leger et al. 2013). The full genome sequences further enabled to study the role of antiviral exoRNAi pathways for vector function of these mosquito species. For example, Keene et al. (2004) were able to show that knockdowns of Dicer and Argonaute genes in *Anopheles gambiae* lead to increased replication of ONNV. However, Carissimo et al. (2015) showed that the induction of the exoRNAi pathway is not essential to control the ONNV infection in the midgut and thus speculate that the role of exoRNAi may be more important during dissemination of the infection than at the initial site of infection. In contrast, Khoo et al. showed that the infection of *Aedes aegypti* with *Togaviridae* is controlled by exoRNAi pathways at the level of the midgut barrier (Khoo et al. 2010). The tissue-specific knockdown of Dcr-2 in the midgut leads to enhanced replication and increased viral escape from the midgut (Khoo et al. 2010). The importance of exoRNAi in the defense of *Aedes aegypti* against SINV was further demonstrated by Campbell et al. (2008b), Myles et al. (2008), and Cirimotich et al. (2009) of which the latter study demonstrated that suppression of the exoRNAi pathway leads to reduced survival of infected mosquitoes. The contradicting observations in two different vector species, *Aedes* and *Anopheles*, indicate that, although exoRNAi is accepted as the major antiviral response in insects (Blair and Olson 2015; Kemp et al. 2013), the importance of this response can be tissue- and vector species-specific. The major role of RNAi in *Aedes aegypti* mosquitoes was further underlined by the observations made by Sanchez-Vargas et al. (2009) showing that DENV is controlled by the exoRNAi pathway and that loss of this pathway leads to increased virus replication and a shortened EIP. Besides *Aedes*, *Culex* mosquitoes are major vectors for arboviruses. Despite their importance, less data on exoRNAi pathway induction and function are available for *Culex* mosquitoes. Brackney et al. (2009) demonstrated that WNV infection induces small RNA production in *Culex quinquefasciatus* mosquitoes indicating that the exoRNAi pathway plays a role in these mosquitoes. Also the production of viRNAs in RVFV-infected *Culex quinquefasciatus* mosquitoes (Dietrich et al. 2017a) and the

demonstration of WNV- and USUV-derived small RNAs in *Culex pipiens* mosquitoes (Fros et al. 2015b) are suggestive for an antiviral role of the exoRNAi pathway in *Culex* spp. However, functional evidence as it is presented for *Aedes* and *Anopheles* mosquitoes is currently lacking for *Culex* mosquitoes.

Besides the exoRNAi pathway, the Piwi-interacting RNA (piRNA) pathway can be activated in mosquitoes after infection with arboviruses. This pathway was initially described in *Drosophila melanogaster*, where the expression of transposons in germline cells and ovarian follicle cells is controlled by piRNAs (Brennecke et al. 2007). The 24- to 29-nt-long piRNAs are generated in a Dicer-independent manner and show a characteristic molecular signature (Brennecke et al. 2007; Morazzani et al. 2012; Vodovar et al. 2012). The piRNA pathway is initiated by the long single-stranded precursor RNAs that transcribed from piRNA clusters in the genome (Brennecke et al. 2007). This signal is amplified by the so-called ping-pong amplification loop (Siomi et al. 2011) including the Argonaute-3 (Ago3), Aubergine (Aub), and Piwi proteins (Brennecke et al. 2007; Gunawardane et al. 2007; Saito et al. 2006). In contrast to *Drosophila melanogaster*, the piRNA pathway has undergone an expansion in aedine and culicine mosquitoes with seven Piwi proteins (Piwi1–7) in *Aedes aegypti* and six Piwi proteins in *Culex quinquefasciatus* (Campbell et al. 2008a; Schnettler et al. 2013). This expansion correlates well with the extended role of the piRNA pathway in mosquitoes. Up to date, virus-specific piRNAs have been found in *Aedes* mosquitoes infected with members of all major arbovirus families and orders *Flaviviridae* (DENV), *Togaviridae* (SINV, CHIKV), and *Bunyavirales* (Dietrich et al. 2017b; Hess et al. 2011; Morazzani et al. 2012; Vodovar et al. 2012). The mechanism by which virus-derived piRNAs are induced is still not completely understood, but a recent study has given some insight into the mechanism of virus-derived synthesis in mosquito cells showing its dependence on Piwi5 and Ago3 proteins (Miesen et al. 2015, 2016). In addition, the Piwi4 protein is shown to be essential to control *Semliki Forest virus* (SFV, *Togaviridae*), BUNV, and RVFV infection in *Aedes aegypti* mosquito cells (Dietrich et al. 2017a, b; Schnettler et al. 2013), and Ago3 is essential to control ONNV in *Anopheles gambiae* (Keene et al. 2004).

The role of the third RNAi pathway, the microRNA (miRNA) pathway in arbovirus infection, is less clear, but recent data point to an involvement of miRNAs in virus-vector interactions (extensively reviewed in Asgari (2014)). The microRNA pathway exists in most metazoans and was initially described as a posttranscriptional regulatory mechanism. The miRNAs are produced by a Dicer enzyme (in insects Dicer-1) and incorporated into RISC-containing Argonaute proteins. This miRNA aids the RISC to a target RNA sequence which is complementary to the 5′8 nucleotides (seed region) of the miRNA. In mammals the role of cellular as well as virus-derived miRNA in modulation of virus replication has been long known (Muller and Imler 2007); however, a lack of knowledge persists on the role of miRNA in arbovirus-vector interactions. After publication of whole genome sequences from *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*, also miRNAs have been identified (*Aedes aegypti* (Li et al. 2009), *Culex quinquefasciatus* (Skalsky et al. 2010), *Anopheles gambiae* (Winter et al. 2007)). A number of studies reported the differential expression of miRNA in these vector mosquitos

after infection with arboviruses. For example, *Culex quinquefasciatus* miR-989 was downregulated, and miR-92 was upregulated during WNV infection, but the meaning of this regulation remains unclear since no target was yet identified for those miRNAs (Skalsky et al. 2010). In *Aedes aegypti*, the infection with DENV serotype 2 alters the abundance of 35 miRNAs of which some have target sequences in genes linked to signal transduction and the cytoskeleton, but to date, no experimental evidence links these potential miRNA-target interactions to virus-vector interactions (Campbell et al. 2014). In contrast, the downregulation of *Aedes albopictus* miR-252 leads to a 1.5-fold increase of DENV serotype 2 virus replication (Yan et al. 2014). Furthermore, *Aedes albopictus* miR-2940, which was found to be unregulated during WNV infection, positively affects WNV replication through the upregulation of metalloprotease m41 *ftsh* (MetP) (Slonchak et al. 2014). However, knockdown of Ago1, the key protein of the miRNA pathway in *Anopheles* and *Aedes*, does not alter replication of several viruses, whereas knockdown of Ago2 (exosiRNA) or Ago3 (piRNA) pathways has a major impact on virus replication. Thus, the role of cellular miRNAs is not entirely clear and needs further investigation.

9.3.1.2 Inducible Antiviral Responses

A couple of inducible mechanisms have been described in *Drosophila* and mosquitoes during the past years. The Toll and immune deficiency (IMD) pathways, initially characterized for their role in the control of bacterial and fungal infections in *Drosophila* (reviewed in Mussabekova et al. (2017)), are now widely recognized immune pathways in mosquitoes (reviewed by Sim et al. (2014)). In mosquitoes, Toll and IMD pathways are induced after pathogen recognition through peptidoglycan recognition proteins (PGRPs). Subsequent intracellular signaling is induced by Spätzle-MyD88 interaction (Toll) or IMD protein (IMD) which leads to the activation of nuclear factor "kappa-light-chain-enhancer" (NF-kB)-like transcription factors, namely, Rel1A (Toll) and Rel2 (IMD). Both pathways trigger the expression of antimicrobial effectors such as cecropins or defensins. The antiviral role of the Toll and IMD pathway was first shown in *Drosophila* after infection with several viruses (Toll, *Drosophila X virus* (Zambon et al. 2005); IMD, SINV and *Cricket paralysis virus* (Avadhanula et al. 2009; Costa et al. 2009)). In mosquitoes first evidence of a potential involvement of the Toll pathway in antiviral defense came from DENV-infected *Aedes aegypti* mosquitoes where 240 genes including key components of the Toll pathway, e.g., Spätzle, Toll, and Rel1A, were differentially regulated (Xi et al. 2008). A functional role of the Toll pathway was further confirmed in DENV-infected *Aedes aegypti* mosquitoes showing that transient Rel1 activation significantly reduces DENV titers, whereas silencing of MyD88 increased virus replication (Xi et al. 2008). Along this line, the induction of the Toll pathway in *Wolbachia*-infected *Aedes aegypti* is believed to be one way how the bacterium interferes with virus replication (Pan et al. (2012); see also section *Wolbachia* below). The impact of Toll pathway activation in other arbovirus infections and other mosquito species is less well studied. SINV and WNV induce the Toll pathway in *Aedes aegypti*

(Colpitts et al. 2011; Sanders et al. 2005), while the latter fails to induce the Toll pathway in *Culex quinquefasciatus* (Bartholomay et al. 2010). Thus, the induction of the Toll pathway due to virus infection might be mosquito species-specific, or orthologues of the Toll pathway have not been completely characterized in other mosquito species, which could explain the lack of detection (e.g., the WNV-induced transcript *CQ G12A2* in *Culex quinquefasciatus* shares 33% homology with the Toll-like receptor of *Aedes aegypti*; Smartt et al. (2009)). The IMD pathway plays a major role in mosquito antibacterial and antiparasite defense (Dong et al. 2009; Garver et al. 2012; Meister et al. 2005). The antiviral role has only been studied recently and in less detail than the Toll pathway. The upregulation of IMD pathway components was shown for *Aedes aegypti* mosquitoes infected with DENV and SINV (Barletta et al. 2017; Luplertlop et al. 2011; Sanders et al. 2005). First indirect evidence for a functional role for the IMD pathway in virus infection was presented by Sim et al. (2013) who showed that silencing of the pathway leads to enhanced viral replication in DENV-refractory strains of *Aedes aegypti*. However, transient activation of the pathway does not influence DENV infection (Xi et al. 2008). Recent findings by Barletta et al. (2017) point to an indirect role of the IMD pathway by controlling the gut microbiota, which then controls SINV replication. Further studies are necessary to clarify the role of the IMD pathway in antiviral defense. Specifically, attention needs to be payed to the clear distinction between the impact of the IMD pathway and the Janus kinase transducer and activator of transcription (JAK-STAT) pathway which both can be activated in mosquitoes by similar stimuli. The insects' JAK-STAT pathway was initially described as a response to stress in *Drosophila* but has been linked with antiviral response in the fly through a microarray study (Dostert et al. 2005). Further evidence of a functional involvement of JAK-STAT pathway in antiviral defense arose from infection experiments of flies with mutations in the Janus kinase gene *hopscotch* (*hop*) with a panel of viruses. These experiments showed that the JAK-STAT pathway is essential to control *Dicistroviridae* (e.g., *Drosophila C virus*) infection in *Drosophila* but is dispensable for antiviral immunity against other viruses tested (Kemp et al. 2013). Bioinformatic analysis of mosquito genome data showed that orthologues of JAK-STAT pathway components, namely, the *domeless* (*dome*) receptor, the *hop* kinase, and STAT transcription factor, are also found in *Anopheles gambiae* and *Aedes aegypti* mosquitoes (Souza-Neto et al. 2009; Waterhouse et al. 2007). Infection of *Aedes aegypti* mosquitoes with DENV significantly induces the JAK-STAT pathway, and silencing of *dome* or *hop* leads to increased virus replication (Souza-Neto et al. 2009; Xi et al. 2008). Furthermore, a recent study by Jupatanakul et al. (2017) demonstrated that genetically engineered mosquitoes overexpressing *dome* and *hop* in the fat body have significantly reduced DENV replication in their bodies and most importantly largely reduced DENV infection rates in the salivary glands. However, the infection rates of ZIKV and CHIKV were not affected in the same mosquitoes. In contrast, Angleró-Rodríguez et al. (2017) demonstrated that ZIKV modulates the expression of Toll-, IMD-, and JAK-STAT-associated genes in *Aedes aegypti* and that the activation of Toll and JAK-STAT pathway significantly reduces ZIKV replication. Thus, it is not clear whether the JAK-STAT pathway is a

pan-flavivirus-specific antiviral pathway similar to what was observed for *Dicistroviridae* in *Drosophila* or whether the antiviral function of this pathway is strictly virus species-specific. Furthermore, it is not clear if JAK-STAT pathway induction has a similar antiviral effect in other mosquito species. Data from WNV infection in *Culex quinquefasciatus* mosquitoes indicate that activation of the JAK-STAT pathway controls virus replication in these mosquitoes. Interestingly, the pathway is activated through secreted Vago, which is induced in a Dicer-2-dependent manner, thereby providing first evidence for a JAK-STAT-RNAi pathway cross talk (Paradkar et al. 2012). In contrast to *Aedes* and *Culex* mosquitoes, *Anopheles gambiae* mosquitoes do not show any transcriptional activation of JAK-STAT or Toll and IMD pathways after experimental infection with ONNV nor did a knockdown of components of this pathway impact ONNV replication (Waldock et al. 2012).

9.3.2 Virus Adaptation to the Mosquito: Immune Evasion and Immune Suppression by Arboviruses

Viruses are constantly exposed to the immune system of their hosts/vectors, which seeks to eliminate viral infection. In consequence, viral pathogens have evolved mechanisms to evade the immune system and infect new vectors.

Genetic reassortment is an important source of antigenic variability for segmented RNA viruses. It allows the fast antigenic shift instead of the slower antigenetic drift and, therefore, is one important factor for the evolution and emergence of viruses with an altered phenotype, disease potential, or host range (Gerrard et al. 2004; Kilian et al. 2013). Extinct or "new" viruses with greater pathogenicity might be created by natural or laboratory reassortment (Briese et al. 2013). An introduction of BUNV (*Orthobunyavirus*, *Peribunyaviridae*) or *La Crosse virus* (LACV) exotic to Europe and the possibility of reassortment with BATV (infraspecies belonging to the *Bunyamwera orthobunyavirus* species and serogroup), respectively, and TAHV (infraspecies belonging to the *California encephalitis orthobunyavirus* species and serogroup) endemic in Europe that may lead to reassortants with greater pathogenicity for humans or other vertebrates have to be considered (Briese et al. 2013; Eiden et al. 2014; Rudolf 2015). Bunyaviruses inherit a tripartite genome consisting of a small (S), medium (M), and large (L) segment. In Africa, NRIV and BUNV have similar geographic distributions across a broad region of sub-Saharan Africa, and both viruses have been isolated from the same species of *Aedes* mosquitoes (Gerrard et al. 2004). Importantly, a large outbreak of hemorrhagic fever in humans in East Africa in late 1997 and early 1998 was related to NRIV, which was found a reassortant of BUNV (S and L segment) and BATV (M segment) (Gerrard et al. 2004). Vector competence studies with *Culex quinquefasciatus*, *Anopheles gambiae*, and *Aedes aegypti* revealed considerable differences in their susceptibility to oral BUNV and NRIV infection. *Culex quinquefasciatus* was refractory and *Anopheles gambiae* moderately susceptible to both viruses. Interestingly, *Aedes aegypti* was moderately susceptible to BUNV but refractory to

NRIV infection (Odhiambo et al. 2014). Therefore, considerable differences in the host range of viruses within the same *Orthobunyavirus* serogroup may occur.

Reassortment is a major driver of rapid evolution in viruses, such as genetic reassortment of avian and human influenza A viruses, bunyaviruses, or bluetongue viruses. Bunyaviruses are considered to originate from strictly inter-mosquito-transmitted viruses, and evolution led to adaptation to vertebrate hosts (Junglen 2016). A marked number of orthobunyaviruses lack the open reading frame encoding nonstructural NSs protein. For example, a novel clade of mosquito-associated bunyaviruses (herbeviruses) and viruses belongs to the *Anopheles A*, *Anopheles B*, and *Tete* serogroups. The orthobunyaviruses that lack the NSs protein fail to prevent induction of interferon-beta mRNA in mammalian cells (Hollidge et al. 2011; Marklewitz et al. 2013; Mohamed et al. 2009; Shchetinin et al. 2015; Weber et al. 2002). Reports about whether NSs may have an effect on virus growth and RNAi in insect cells are controversial (Hart et al. 2009; Hollidge et al. 2011; Rudolf 2015). The phenotype of closely related virus strains of the same species may even differ in their route of transmission. An originally strictly inter-arthropod-transmitted virus circulating within arthropod populations may convert to an arthropod-borne virus (may be due to evolutionary advantages, faster temporal and spatial distribution, and therefore higher fitness) that is more efficiently transmitted using an intermediate amplifying host, e.g., vertebrate hosts. Furthermore, a change in phenotype from a strictly inter-arthropod-transmitted virus toward a virus that may be directly transmitted between mammals cannot be precluded, as previously described for the two atypical bluetongue virus (BTV) serotypes 26 and 27 (Batten et al. 2014; Bréard et al. 2018).

In nature, reassortment may occur in the vertebrate host or the mosquito vector. However, studies of genome reassortment of orthobunyaviruses in vertebrates were unsuccessful (Beaty et al. 1985). In contrast, reassortment of heterologous as well as homologous orthobunyaviruses (*Peribunyaviridae*, *Bunyavirales*) was demonstrated in the mosquito vector (Borucki et al. 1999). In *Aedes triseriatus* mosquitoes, 20% of the offspring transovarially infected with LACV became superinfected when challenged with a second LACV strain or the serologically closely related *Snowshoe hare virus* (SSHV) (Borucki et al. 1999). Furthermore, a greater viral intra-host diversity was detected in ticks infected with *Crimean-Congo hemorrhagic fever orthonairovirus* (CCHFV; another member of the *Bunyavirales* order) compared to the vertebrate host (Xia et al. 2016) suggesting the arthropod vectors are the primary source of antigenic shift and drift. Accordingly, there is an urgent need to continually monitor emergent arboviral genotypes circulating within particular regions as well as vectors mediating these transmissions to preempt and prevent their adverse effects, genetic mechanism for species specificity, and vector competence owing to reassortment that needs further investigation (Odhiambo et al. 2014).

Besides such drastic measures as exchange of genome segments, viruses have developed other mechanisms to avoid or circumvent the vector and host immune systems. A study by Brackney et al. (2009) showed that siRNAs generated from WNV genomes in *Culex* mosquitoes mostly matched specific “hot spot” regions in the virus genome. These specific regions were more prone to mutations than other so-called cold spots, indicating that an enhanced mutation rate is one mechanism to

escape siRNAs targeting. Also, abundantly expressed sub-genomic RNAs derived from the highly structured RNA encoded in the 3′ untranslated region of all flaviviruses (sub-genomic flavivirus (sf) RNAs) have been described (Pijlman et al. 2008). The sfRNAs derived from DENV and WNV genomes during infection have been shown to suppress Dcr-2-dependent dsRNA cleavage most likely through a direct inhibition of Dcr-2 (Moon et al. 2015; Schnettler et al. 2012). Furthermore, this inhibition in mosquito immunity by sfRNAs is curtail for successful virus transmission as shown by decreased transmission of sfRNA-deficient WNV in *Culex* mosquitoes and enhanced transmission of sfRNA overexpressing DENV in *Aedes aegypti* (Moon et al. 2015; Pompon et al. 2017).

Looking at classical viral suppressors of RNA silencing proteins (VSRs), diverse examples with multiple modes of action can be found. However, some common themes were established during evolution. The NSs protein of the plant-infecting *Tomato spotted wilt orthotospovirus* (TSWV; *Tospoviridae*, *Bunyavirales*), the B2 protein of the insect *Flock house virus* (FHV; *Nodaviridae*), and the VP3 protein of the mosquito-specific *Culex Y virus* (CYV; *Birnaviridae*) all sequester dsRNA molecules of different lengths and by this inhibit the recognition of these RNA molecules by Dcr-2 and incorporation of small dsRNA species into RISC, respectively. For arboviruses, not a lot of those classical VSRs have been identified; some authors even speculate that arboviruses do not express VSRs as matter of adaptation to avoid undue replication of the virus in the mosquito vector. Nevertheless, the DENV NS4B protein was shown to have VSR activity. While it could not bind to dsRNAs, it interferes with dicing (Kakumani et al. 2013).

9.3.3 *Impact of* Wolbachia *on Vector Competence and Virus Replication*

Several studies during recent years have shown that *Wolbachia*, a family of endosymbiotic *Alphaproteobacteria*, are associated with resistance to viral infection in several insect species including *Drosophila* (Hedges et al. 2008) and mosquito species (Glaser and Meola 2010; Moreira et al. 2009). *Wolbachia pipientis* was first described in the mosquito *Culex pipiens* and is inherited maternally via egg cytoplasm. The effects of *Wolbachia* infection in mosquitoes are widespread and somewhat contradictory. Several studies describe inhibitory effects on the infection with pathogens, especially in mosquitoes that have been artificially infected. However, others have reported no effect on virus infection or even enhanced virus infection rates (reviewed in Johnson (2015)).

Concerning the *Culex* complex, up to now, reported observations are contradictory. Glaser and Meola described an enhanced resistance of *Wolbachia*-infected *Culex quinquefasciatus* toward WNV (Glaser and Meola 2010), whereas Dodson et al. (2014) reported an enhancement of WNV replication in artificially *Wolbachia*-infected *Culex tarsalis* mosquitoes. Furthermore, the resistance phenotype in *Culex quinquefasciatus* as well as *Culex pipiens* toward WNV is not only dependent on

the presence or absence but also on the *Wolbachia* density (Micieli and Glaser 2014). In *Aedes* mosquitoes, repression of virus infection but again lack of effect on virus replication due to *Wolbachia* infection was observed. The most impressive phenotypes were observed in *Aedes aegypti*, which is one of the few mosquito species not naturally infected with *Wolbachia*. Artificial transfection of the *Drosophila wMel* and *wMelPop* strains leads to severe reduction of virus replication, dissemination, and transmission of DENV, YFV, CHIKV, and WNV (Hussain et al. 2013; Moreira et al. 2009; van den Hurk et al. 2012; Walker et al. 2011). The transinfection with the *wAlbB* strain from *Aedes albopictus* also reduced DENV infection (Bian et al. 2010). However, naturally *Wolbachia* (*wAlbA* and *wAlbB*)-infected *Aedes albopictus* did not show virus repression phenotypes when transinfected with the *Drosophila*-specific *Wolbachia* strain *wMel*. Only on rare occasions, i.e., when Blagrove et al. (2012) replaced the natural *Wolbachia* strain by the *wMel* strain, they were able to observe reduced transmission efficiency in those mosquitoes for DENV.

The mechanisms underlying the resistance phenotype are poorly understood. Some studies link the effect of *Wolbachia* infection in mosquitoes to immune priming. In *Drosophila* several studies argue against an involvement of Toll an IMD pathway priming, since Rancès et al. (2013) showed that priming of these pathways is not necessary for the *Wolbachia*-mediated blocking of DENV and Chrostek et al. (2014) found a high antiviral protection without immune upregulation after interspecies transfer of *Wolbachia.* Most studies analyzing the resistance phenotype in mosquitoes used *Aedes aegypti* mosquitoes and DENV infection. This combination has shown the most pronounced resistance phenotype, which might be due to the fact that *Aedes aegypti* mosquitoes are not naturally infected with *Wolbachia*. The activation of the Toll, IMD, JAK-STAT, and melanization pathways in *Aedes aegypti* was investigated among others by Kambris et al. (2009). Other studies have demonstrated the crucial role of the Toll pathway to control DENV infection in these mosquitoes (Xi et al. 2008). Hence, it is rational to suspect a causative link between the blocking phenotype and immune activation. Indeed, such a link could be established by Pan et al. (2012). Recent data collected from *wAlbB*-infected *Aedes aegypti* mosquitoes even show that the permanent activation of the Toll and IMD pathways by *Wolbachia* is needed by the bacteria to establish a stable infection in the mosquito (Pan et al. 2018). However, the same is not the case in the natural DENV vector *Aedes albopictus.* An additional transinfection with the heterologous *wMel* strain does not lead to significant upregulation of the innate immune pathways (Blagrove et al. 2012). A recent study with *Wolbachia*-infected *Aedes aegypti* cell lines demonstrated that the induction of RNAi, Toll, and IMD pathways must not necessarily be the cause of the protective effect since only the knockdown of the RNAi pathway leads to a small but significant reduction of the protective phenotype (Terradas et al. 2017). It has been demonstrated that a Vago homolog in *Aedes aegypti* (*AedesVago1*) inhibits the replication of DENV (Asad et al. 2017). This is of special interest, since Vago has been demonstrated to be induced in a Dcr-2-dependent manner in *Culex quinquefasciatus* and facilitated crosstalk between the exoRNAi pathway and the JAK-STAT pathways. Taken together, all data on immune priming and the inhibition phenotype induced by

Wolbachia show that immune priming might explain this effect in parts. However, other mechanisms need to be considered to fully explain the inhibition phenotype. A couple of studies create possible links between insect cell physiology and infection resistance. The infections of *Aedes aegypti* cells with the *Wolbachia* strain *wMelpop* leads to a downregulation of MCT1 expression (Osei-Amo et al. 2012). Since alteration of MCT expression has been shown to induce apoptosis in insects (Jang et al. 2008), there might be a link between enhanced apoptosis and reduced virus replication. Also the energy metabolism of cells is discussed as possible cause for virus inhibition. DENV is known to manipulate the cellular fatty acid biosynthetic pathway to create a favorable environment for viral replication complexes at intracellular membranes (Perera et al. 2012). It has been also shown that *Wolbachia* requires unsaturated fatty acids from host cells, since it cannot synthesize those. Thus, both bacteria and viruses need unsaturated fatty acid from the host; by limiting this resource, bacterial growth could suppress virus replication. Cholesterol was also shown to be crucial for DENV and alphavirus replication (Hafer et al. 2009; Lu et al. 1999; Rothwell et al. 2009) as well as for *Wolbachia* replication. A high growth rate of *Wolbachia* could deplete the insect cells from cholesterol and by this block the virus from essential resources for its replication (Moreira et al. 2009). This hypothesis was also confirmed in *Drosophila* when a cholesterol-enriched diet reduced the protective effect of *Wolbachia* against virus infection (Caragata et al. 2013). In *Aedes aegypti* cells, treatment with 2-hydroxypropyl-β-cyclodextrin to restore cholesterol homeostasis rescued DENV replication (Geoghegan et al. 2017). To confirm this mechanism in different mosquito and virus species, future studies will be necessary.

9.4 Conclusions

It can be summarized that virus replication in vector mosquitoes and thus the emergence of arbovirus infection are controlled by a myriad of different factors, including the mosquito abundance, temperature profiles, habitats, abundance of susceptible hosts, and other external factors. Also, the importance of intrinsic factors including the immune system of the insect and the presence of microbiota and endosymbionts such as *Wolbachia* cannot be stressed enough. The available data draw a complicated picture of the importance of all different factors which all warrant further research for clarification, altogether making vector competence studies a challenging but no less fascinating future topic.

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Chapter 10
Mosquitoes and the Risk of Pathogen Transmission in Europe

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Abstract Worldwide, mosquitoes are known as nuisance biters and disease-transmitting vectors causing about one million deaths annually. In Europe, around 100 mosquito species have been described with the medically most relevant species belonging to the genera *Aedes*, *Anopheles* and *Culex*. Due to several climatic and non-climatic factors, some mosquito species as well as pathogens were either newly introduced or reintroduced to Europe within the last decades and have been causing different outbreaks of diseases. Currently, the risk of an infection with a mosquito-borne disease like dengue and chikungunya fever is higher in Mediterranean countries than in the north of Europe and can be partly ascribed to the establishment of *Aedes albopictus*, which is one of the most invasive mosquito species worldwide and a competent vector species for several diseases. Ecological niche modelling implies that *Aedes albopictus* could spread further north with the warming climate in the future. As species monitoring is cost and time-consuming, ecological niche modelling could help identify potential new habitats for mosquito establishment, in order to better target areas for monitoring and surveillance programmes to those at greatest risk. However, mosquito species native to Europe, like members of the *Culex pipiens* complex, can also act as vectors for pathogens like the West Nile virus and therefore should also be considered in those programmes.

Keywords Ecological niche modelling · Europe · Health threat · Invasive mosquitoes · Pathogens · Vector-borne disease

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10.1 Introduction

Every day, the world's population faces a multitude of risks with infectious diseases being one of the major health threats. Especially diseases, which are transmitted through vector species, are a major concern. These vectors are almost exclusively arthropods, of which mosquitoes are the best known disease vectors, and of particular importance because of their serious health risks for humans and animals (Semenza and Menne 2009; AMCA 2017; WHO 2017).

Today, about 3500 mosquito species are described worldwide, but only some of them are known to be competent vectors (Becker et al. 2014; ECDC 2014; CDC 2015). For example, of nearly 430 known *Anopheles* species, only 60 are able to transmit disease-causing agents like *Plasmodium* spp. (WHO 2014). The medical relevance of mosquitoes has been known since the late nineteenth century as Sir Patrick Manson discovered an important lifecycle step of the filarial worm *Wuchereria bancrofti* within the mosquito *Culex pipiens quinquefasciatus* (Lehane 2005). In the following years, the transmission of disease-causing agents by mosquitoes was shown for avian malaria (*Plasmodium* spp.), yellow fever as well as dengue fever (Ross 1897; Reed et al. 1900; Graham 1902; Lehane 2005). The drainage of wetlands as well as the massive use of insecticides between 1940 and 1950 had led to a reduction in the mosquito populations. As a result, infections with yellow fever virus or *Plasmodium* spp. were significantly reduced in the 1960s and were no longer a health problem in many areas outside of Africa (Gubler 1998; WHO 2014). In 1973, Europe was declared malaria-free (Krüger et al. 2014). However, after the successful reduction of vector populations, control programmes and prevention were discontinued, which led to a new increase in vector-associated infectious diseases from the 1970s on (Gubler 1998; WHO 2014). Today, more than 50% of the world's population live in vector-borne disease risk zones, mostly in low-income countries (WHO 2014). Due to several factors, many pathogens and mosquitoes have expanded their distribution again or might become an (re-)emerging health and societal problem even in high-income countries (Zeller et al. 2013; Becker et al. 2014). Climate change and global transportation are the most common reasons for the further spread of vectors and pathogens beyond their native ranges. However, many other factors are important for species and disease agents to expand their ranges successfully (see risk assessment). In this chapter, the potential threats by mosquito-borne pathogens in Europe as well as the possible drivers of future changes in their distribution are discussed. We also show how ecological niche modelling could help to improve current monitoring and surveillance programmes using the invasive mosquito species *Aedes albopictus* as an example.

10.2 Mosquitoes as Disease Vectors in Europe

Around the world, mosquitoes are known as nuisance biters and disease-transmitting vectors. For Europe, nearly 100 (including five non-native) species have been described of which about 29 species could be involved in the transmission of

pathogens (Becker et al. 2010; ECDC 2014). The most medically relevant species in Europe belong to the genera *Anopheles* (*An.*), *Culex* (*Cx.*) and *Aedes* (*Ae.*) (Becker et al. 2014; ECDC 2014; Calzolari 2016). While *Anopheles* species, like widely distributed *An. plumbeus,* are susceptible for malaria parasites, members of the *Cx. pipiens* complex are suspected vectors for the West Nile virus (Fonseca et al. 2004; Farajollahi et al. 2011; Schaffner et al. 2012). Due to their opportunistic feeding behaviour as well as their ability to form hybrids, mosquitoes of the *Cx. pipiens* complex could also act as bridge vectors (Farajollahi et al. 2011; Becker et al. 2014).

However, not only the species native to Europe but also non-native species might become a threat to public health in the future. Among them, five recently introduced *Aedes* species have gained attention in recent decades: *Ae. aegypti*, *Ae. albopictus*, *Ae. atropalpus*, *Ae. japonicus* and *Ae. koreicus* (Schaffner et al. 2013; Medlock et al. 2015; ECDC 2017a, b). All of these species have most likely been introduced to Europe via global transport and trade, especially by shipping of their eggs in used tyres and 'lucky bamboo' (Peyton et al. 1999; Versteirt et al. 2012; Medlock et al. 2015; ECDC 2017c). Due to their unintentional introduction, some of the species have been able to widely spread in Europe (Medlock et al. 2015; ECDC 2017c, d, e, f). Currently, about 45% of the European population lives in areas with a high potential for invasive mosquito species' establishment and a related risk of pathogen transmission (Petrić et al. 2014).

It is also assumed that these species will spread even further into new regions in Europe due to global warming (Beierkuhnlein 2007; Kowarik 2010; Cunze et al. 2016a). Although the vector competence of all these five *Aedes* species has been confirmed in the laboratory for several (human) pathogens, *Ae. atropalpus, Ae. japonicus* and *Ae. koreicus* are not considered important vectors in the field in their native range (Schaffner et al. 2013; Medlock et al. 2015; ECDC 2017c).

In contrast to this, a main health threat can be associated with the introduction and establishment of the yellow fever mosquito *Ae. aegypti* and the Asian tiger mosquito *Ae. albopictus* (Avšič-Županc 2013; Medlock et al. 2015). Both species are confirmed vectors for several pathogens under laboratory conditions as well as in the field and readily feed on human hosts (Medlock et al. 2015; ECDC 2017f, g).

Apart from the name-giving yellow fever, *Ae. aegypti* is one of the main vector species for dengue fever, chikungunya and Zika virus (Schaffner et al. 2013; Medlock et al. 2015; ECDC 2017f). Originally from Africa, *Ae. aegypti* has become one of the globally most widespread mosquito species. Between the eighteenth and twentieth century, it was widely established in Southern Europe where it was associated with large epidemics of dengue and yellow fever (Eritja et al. 2005; Jansen and Beebe 2010; Schaffner et al. 2013; Medlock et al. 2015; ECDC 2017f).

At present, established populations of *Ae. aegypti* are being recorded in eastern regions of the Black Sea (ECDC 2017a). A further spread of the species in Europe is currently limited due to the cold winter temperatures and the associated mortality of the eggs (Otero et al. 2006; Gould and Higgs 2009; Jansen and Beebe 2010). However, climatic changes projected for the future are assumed to promote an expansion and might result in a re-establishment in the Mediterranean area (ECDC 2017f).

Whereas cold temperatures seem to set limits to the spreading of *Ae. aegypti*, the Asian tiger mosquito *Ae. albopictus* possesses a high ecological plasticity and has

the ability to adapt to cold temperatures (Paupy et al. 2009; ECDC 2017g). *Aedes albopictus* is distributed further north than *Ae. aegypti* in Europe and is listed among the top 100 most invasive species worldwide (Paupy et al. 2009; Medlock et al. 2015; ISSG 2017; ECDC 2017g). With its native range in Asia, the species was observed in Albania in 1979 for the first time and has been reported from at least 16 European countries since then (Buhagiar 2009; Paupy et al. 2009; Reiter 2010).

Besides its strong invasive ability, the species has the ability to transmit a variety of pathogens, e.g. 26 arboviruses (e.g. dengue, chikungunya or Zika virus) or different dirofilarial worms (Cancrini et al. 2003a, b, 2007; Gratz 2004; Paupy et al. 2009). Its high vector competence was not only shown under laboratory conditions but was also detected in the field (Mitchell 1991; Paupy et al. 2009; Koch et al. 2016). Moreover, due to its opportunistic feeding behaviour, the species serves as a bridge vector (Eritja et al. 2005; Paupy et al. 2009).

Like all insects, mosquitoes can develop faster at higher temperatures. Thus, projected climate warming may not only lead to establishment in areas where temperatures were not suitable for survival before (see chapter on modelling) but also to increases in population sizes (Medlock and Leach 2015).

10.3 Pathogens Transmitted by European Mosquitoes

Pathogens transmitted by mosquitoes comprise viruses, bacteria, nematodes and protozoa. More than one million people die annually because of an infection with vector-borne diseases (WHO 2014). Many mosquito species are vector competent not only for one pathogen (see *Ae. albopictus*). Worldwide, about 13 different disease agents transmitted by mosquitoes are relevant for human and animal health. In Europe, especially *Dirofilaria repens*, *Dirofilaria immitis*, Usutu virus, Sindbis virus, chikungunya virus, dengue virus and the West Nile virus are relevant (ECDC 2014). In most cases, the mosquito vectors are infected during a blood meal, but horizontal transmission (involving, e.g. transovarial or mating processes) is also possible. Once infected, most of the vector species remain infective for the rest of their lifetime (Calzolari 2016). Once absorbed into the mosquito body, pathogens take different routes within the body to propagate, develop and await their transition into a new host.

10.4 Recent Outbreaks of Mosquito-Borne Diseases in Europe

During the last decades, Europe has been facing different outbreaks of diseases categorized into tropical or subtropical diseases (e.g. chikungunya fever, Zika fever), re-emerging diseases (dengue fever, malaria) or diseases introduced to different countries (e.g. West Nile fever, dirofilarial worms) (Zeller et al. 2013). Taken

Table 10.1 Malaria cases in Europe (ECDC 2017h)

	2008	2009	2010	2011	2012	2013	2014	2015
Austria	57	44	48	7	28	42	68	81
Belgium	181	144	166	184	206	253	235	277
Bulgaria	0	8	5	8	16	8	10	20
Croatia	.	.	.	.	.	0	6	7
Cyprus	0	1	1	6	1	3	8	3
Czech Republic	22	10	11	28	25	27	30	29
Estonia	0	4	1	1	6	3	3	4
Finland	42	34	33	33	46	38	39	39
France	2246	2199	2439	1891	1851	2166	2298	2500
Germany	547	523	615	562	547	637	.	.
Greece	39	51	45	92	95	25	38	84
Hungary	5	8	5	10	5	5	15	12
Ireland	82	.	90	82	61	71	79	82
Italy	586	651	662	.	.	.	.	.
Latvia	2	6	5	4	3	4	6	1
Lithuania	3	3	3	3	6	8	5	8
Luxembourg	2	3	12	3	7	3	3	1
Malta	3	1	0	1	2	5	3	7
Netherlands	229	237	247	253	194	162	276	340
Norway	32	34	37	30	37	72	120	94
Poland	22	22	35	14	21	36	19	29
Portugal	42	44	50	67	71	117	144	194
Romania	13	12	19	40	32	43	47	30
Slovakia	2	0	2	1	6	4	5	0
Slovenia	3	7	9	6	7	3	7	5
Spain	290	356	351	405	421	518	688	706
Sweden	91	81	115	95	85	119	354	250
United Kingdom	1371	1495	1761	1677	1378	1501	1510	1397
EU/EEA	5912	5978	6767	5503	5157	5873	6016	6200
Autochthonous cases	64%	23%	19%	1%	0.5%	0	0	0

together, these pathogens are responsible for over 9000 infections annually in Europe (see Tables 10.1, 10.2, 10.3, 10.4, and 10.5) (ECDC 2017h). As already mentioned above, malaria is not new to Europe. Partly eradicated in the 1960s, cases continued to rise again during the next 50 years (Nájera et al. 2011). Over the last 10 years, the rate of malaria infections in Europe remained stable with 5000–6000 cases per year (see Table 10.1). In 2014, nearly all cases can be related to travel activity, for example, people got infected during their holidays in areas where malaria is endemic (ECDC 2017h). However, there are also some autochthonous cases, e.g. three cases in Spain and two in France (2014), six infections with *Plasmodium vivax* in Greece (2015) and four infections in Italy (2017) (HCDCP 2015; ECDC 2017h). Another disease not appearing for the first time in Europe is the West Nile fever. First described from Uganda in 1937, it is causing sporadic

Table 10.2 West Nile fever cases in Europe (ECDC 2017h)

	2008	2009	2010	2011	2012	2013	2014	2015
Austria	.	.	1	.	.	.	1	7
Bulgaria	.	.	.	.	2	.	.	3
Croatia	.	.	.	.	5	16	.	.
Czech Republic	.	.	.	.	.	1	.	.
France	.	.	.	.	.	.	.	1
Greece	.	.	262	100	161	86	15	.
Hungary	19	7	18	.	17	31	11	18
Italy	3	.	3	14	50	69	.	.
Portugal	.	.	.	.	.	.	.	1
Romania	2	2	57	11	14	24	23	32
Slovenia	.	.	.	.	.	1	.	.
Spain	.	.	2	.	.	.	.	.
EU/EEA	24	9	343	125	249	212	50	
(No information regarding autochthonous cases)								

Table 10.3 Dengue fever cases in Europe (ECDC 2017h)

	2008	2009	2010	2011	2012	2013	2014	2015
Austria	0	0	11	0	2	89	91	104
Belgium	60	53	129	41	73	139	110	108
Croatia	.	.	.	.	1	3	2	.
Estonia	0	0	0	0	0	0	9	12
Finland	35	35	50	45	90	80	38	54
France	56	64	596	55	110	271	212	167
Germany	273	298	595	288	616	878	626	.
Greece	0	0	0	0	0	1	4	2
Hungary	6	1	7	2	3	10	6	12
Iceland	.	0	0	0	0	0	0	0
Ireland	0	0	0	0	7	15	21	8
Italy	12	10	51	44	74	142	79	103
Latvia	0	1	8	2	7	7	1	4
Lithuania	0	0	0	1	0	1	3	9
Luxembourg	0	0	2	1	0	0	0	0
Malta	0	0	1	0	0	0	0	1
Netherlands	.	.	.	.	.	.	3	18
Norway	.	.	.	.	30	57	73	98
Poland	2	4	6	5	5	13	15	12
Portugal	.	.	.	.	.	.	.	14
Romania	1	0	0	2	3	6	6	7
Slovakia	0	0	0	0	3	4	0	2
Slovenia	6	4	8	8	10	8	2	3
Spain	0	4	0	0	0	0	.	168
Sweden	73	100	151	103	175	220	119	159
United Kingdom	6	3	7	13	0	571	376	423
EU/EEA	530	577	1622	610	1209	2515	1796	1488
Autochthonous cases	16.5%	19%	10%	0	0	0	0.2%	2.5%

Table 10.4 Chikungunya fever cases in Europe (ECDC 2017h)

	2008	2009	2010	2011	2012	2013	2014	2015
Austria	0	8	2	2	0	0	.	.
Belgium	0	6	8	8	6	7	74	44
Czech Republic	0	0	0	0	0	0	3	1
Finland	0	3	1	0	0	1	4	7
France	1	13	44	12	6	11	550	52
Germany	17	54	37	13	9	16	162	.
Greece	0	0	0	0	0	0	1	0
Hungary	0	0	0	0	0	0	2	2
Ireland	0	0	1	0	0	0	1	1
Italy	9	3	7	2	5	3	39	18
Latvia	0	0	0	0	0	0	0	2
Netherlands	.	.	.	.	.	.	33	24
Spain	5	6	0	4	2	2	272	234
Sweden	.	0	0	0	2	6	19	23
United Kingdom	9	56	79	14	21	26	301	106
EU/EEA	41	149	179	55	51	72	1461	514
Autochthonous cases	2.5%	9%	1%	0	0	0	1	0.2%

Table 10.5 Zika fever cases in Europe (ECDC 2017h)

	2015	2016	2017
Austria	1	41	7
Belgium	1	120	33
Czech Republic	.	13	2
Denmark	.	8	6
Finland	1	6	1
France	.	1141	23
Greece	.	4	1
Hungary	.	2	0
Ireland	1	15	2
Italy	.	101	.
Luxembourg	.	2	.
Malta	.	2	.
Netherlands	11	98	5
Norway	.	8	4
Portugal	.	18	0
Romania	.	3	0
Slovakia	.	3	0
Slovenia	.	7	0
Spain	10	301	34
Sweden	1	34	14
United Kingdom	3	194	8
EU/EEA	29	2121	140
(No autochthonous cases)			

outbreaks in Europe since the 1960s (George et al. 1984; Hubalek and Halouzka 1999). The largest outbreak was in 1996–1997 in Romania with about 500 clinical cases (Hubalek and Halouzka 1999). In 2010, an outbreak occurred in northern Greece which caused 33 deaths (Danis et al. 2011) as well as in southern and central Romania leading to 5 deaths (Sirbu et al. 2011). In 2012, an outbreak of West Nile fever was reported in north-eastern Italy which caused two fatalities (Barzon et al. 2013). Infections with the West Nile virus are reported every year in the EU (see Table 10.2).

Like the West Nile virus, the dengue virus also caused several epidemics in Europe over the last decades (see Table 10.3). In 2010, two autochthonous cases were detected in southern France (La Ruche et al. 2010). In the same year, two more transmission events with one, respective 15 cases occurred in Croatia (Schmidt-Chanasit et al. 2010; Gjenero-Margan et al. 2011). In 2013, five autochthonous cases of dengue fever were identified in southern France (Marchand et al. 2013) and four in south-eastern France (Giron et al. 2015).

The chikungunya virus was formerly known as a tropical pathogen and now expands its distribution. During 2007, the first autochthonous outbreak of chikungunya fever in a non-tropical area was reported from Italy with 205 infections and 1 death (Rezza et al. 2007). In 2010, an outbreak of chikungunya fever was reported in south-eastern France with two confirmed cases (Gould et al. 2010). Since the last decade, infections with chikungunya virus have been reported every year (see Table 10.4). Both the transmission of dengue and chikungunya viruses are associated with the arrival of the non-native vector species *Ae. albopictus* (Rezza et al. 2007; Bonilauri et al. 2008; Gould et al. 2010; Marchand et al. 2013). Since 2015, infections with the Zika virus are reported regularly from Europe but without autochthon cases yet (see Table 10.5).

Other viruses with lower public attention are the Sindbis virus and the Usutu virus. Sindbis fever outbreaks in Europe have been occurring most often in Finland with intervals of approximately 7 years (1974, 1981, 1988, 1995), although recent cases deviate from this rule (Kurkela et al. 2008). The last two outbreaks occurred in 2002 and one in 2012, with approximately 200 infection cases. The last European outbreak was in northern Sweden in 2013. However, the virus has also been found in mosquitoes from southern Germany and in one crow in Berlin (Germany) as well as in birds in the United Kingdom and the Czech Republic (Adouchief et al. 2016).

The first emergence of the Usutu virus in Europe can be dated back to 1996 when it was detected in Italy (Mani et al. 1998). In 2001, it caused mass mortality of *Turdus merula* in Austria (Weissenböck et al. 2013). Subsequently, it was found in the United Kingdom (2001) (Buckley et al. 2006), Hungary (2003) (Chvala et al. 2007), Switzerland (2006) (Steinmetz et al. 2011), Spain (2006) (Busquets et al. 2008), Germany (2011) (Becker et al. 2012) and Belgium (Garigliany et al. 2014) and was identified in bats, birds and mosquitoes.

In addition to infections with *Plasmodium* spp. and different viruses, the transmission of different filarial worms is increasingly reported. Morchón et al. (2012) have shown that the distribution of the dog heartworm (*Dirofilaria immitis*) is slowly shifting northwards in Europe. About 10% of clinics in nonendemic areas report an

increase in infection cases (Genchi et al. 2014), although the overall number of cases is still very low. Similar to their sister species, *Dirofilaria repens* occurs only very rarely in Europe. One infection case was reported in 2008 from Slovakia (Babal et al. 2008), five cases occurred in the Czech Republic in 2010 and 2014 (Matějů et al. 2016), and three cases were recorded from Poland between 2009 and 2011 (Cielecka et al. 2012). In Germany, one case of infection with *D. repens* was reported in 2014 (Tappe et al. 2014).

Promoted by ongoing climate change and human activities, vectors and pathogens may be able to expand their potential range towards more temperate climate zones into higher latitudes in the future (Romi et al. 2008; Scholte et al. 2008; Roiz et al. 2011; Cunze et al. 2016a; Koch et al. 2016; ECDC 2017g) (Fig. 10.1).

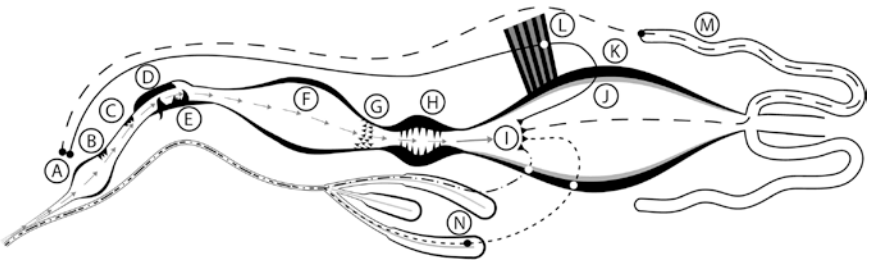

Fig. 10.1 Schematic (non-proportional) illustration of pathogen pathways within a mosquito's digestive system (illustration and description modified after McGreevy et al. 1978 and Beerntsen et al. 2000). All pathogens entering the mosquito's digestive system during a blood meal must pass several apertures and defensive mechanisms before entering the midgut (I). They are sucked in with the cibarial pump (A); pass the palatal papillae (B), dorsal papillae (C), posterior hard palate (D), ventral papillae and cibarial armature (E); and are pushed further with the pharyngeal pump (F) along the pharyngeal armature (G) and the proventriculus (H), ending in the midgut (I). **Filarial worms**, responsible for several human diseases (e.g. *Dirofilaria repens*), (solid line), penetrate the midgut epithelium (K) and migrate into a thoracic muscle (white dot, L) where they develop into third instar larvae. After that, they break out of the muscle, enter the haemocoel and move towards the head area (black dot) where they pause and only actively emerge, while the mosquito is sucking blood. They then move into the wound left behind by the mosquito's proboscis. The dog's heartworm (*Dirofilaria immitis*) (long-dashed line) moves through the midgut into the Malpighian tubules, enters the distal cells thereof and develops into third instar larva (black dot). Similar to their sister species, they undergo a phase of development, break out of the Malpighian tubules and move towards the head region, where they emerge the same way as *Dirofilaria repens*. **Viruses** (short-dashed line) enter the midgut epithelial cells (white dot, K), replicate, exit the cells and pass through the haemocoel to the salivary glands (N), where they replicate again (black dot) and stay dormant until they are injected into a new host. **Protozoan parasites** like malaria-causing *Plasmodium* species (dot-dashed line) linger within the midgut for a few hours and undergo syngamy to form their ookinetes. Afterwards, they migrate through the peritrophic matrix (J) and pass through the midgut epithelium where they embed themselves between the epithelium and midgut basal membrane (white dot). Here, they undergo sporogony in the oocysts and produce sporozoites. When mature, the sporozoites burst out of the oocyst, travel through the haemocoel, penetrate the salivary glands and remain there until they are injected into a host. In consequence of these various possible pathways, multiple pathogens can be transmitted by one mosquito at once (Rückert et al. 2017) or by multiple mosquito species, enabling a widespread of these infections

10.5 Modelling of Potential Current and Future Distributions of *Aedes albopictus*

Within the last few years, different European countries have started monitoring and surveillance programmes with the aim of recording the occurrence of native and non-native mosquito species and investigating their vector abilities (e.g. Kampen et al. 2015). One famous example for a vector species formerly found only in tropical and subtropical regions but now considered an invasive species in Europe is *Aedes albopictus* (e.g. Bonizzoni et al. 2013). This species has established in large parts of Southern Europe; it shows a high invasive potential and is of high medical relevance due to its vector competency for many pathogens (reviewed in Koch et al. 2016). Therefore, a European-wide monitoring of the spread of this species is urgently needed, although this is considered to be quite time-consuming (Medlock et al. 2015). One approach that may be applied at this point is the use of ecological niche models (ENMs). By means of these models, areas with suitable climatic conditions for the species, thus, potentially endangered by a new establishment of the species outside the former range, can be identified (Escobar and Craft 2016). Ecological niche models are correlative statistical approaches used to estimate the habitat suitability for a species in certain regions. They are based on species' occurrence data as well as information on the environmental conditions prevailing in the study area (e.g. Elith et al. 2010). In the example presented here (Figs. 10.2, 10.3, and 10.4), an ensemble forecasting for *Ae. albopictus* based on ten commonly used algorithms was applied (Cunze et al. 2016a). Ensemble forecasting refers to the integration of different models into one 'consensus' model. Consensus models have been found to provide rather robust estimates of climatic suitability (Araújo and New 2007). As explaining variables six climatic variables were used (mean temperature of coldest quarter, mean temperature of warmest quarter, temperature annual range, annual mean precipitation, precipitation in warmest quarter and precipitation seasonality) and were provided by WorldClim (www.worldclim.org). Occurrence records (n = 336) were taken from Kraemer et al. (2015a, b) and Koch et al. (2016). The climatic habitat suitability was projected under different climatic conditions considering four different time periods. The scenario under current climatic conditions comprises the average of conditions between 1960 and 1990, whereas the future scenarios refer to a period of 20 years each: 2030 (average between 2021 and 2040), 2050 (average between 2041 and 2060) and 2070 (average between 2061 and 2080). Data on future conditions was taken from the fifth Intergovernmental Panel on Climate Change (IPCC) Assessment Report (AR5) (IPCC 2013). These data are according to the RCP 4.5 scenario, which assumes a global rise of annual greenhouse gas emissions until 2040 and then a decline until the end of the twenty-first century (Meinshausen et al. 2011) resulting in a moderate increase of temperatures.

For each considered time period, different areas were identified to be climatically suitable for *Ae. albopictus*. Figure 10.2 combines the modelling results and displays trends (stable climatic suitability, new climatic suitability, loss of climatic suitability) in different colours.

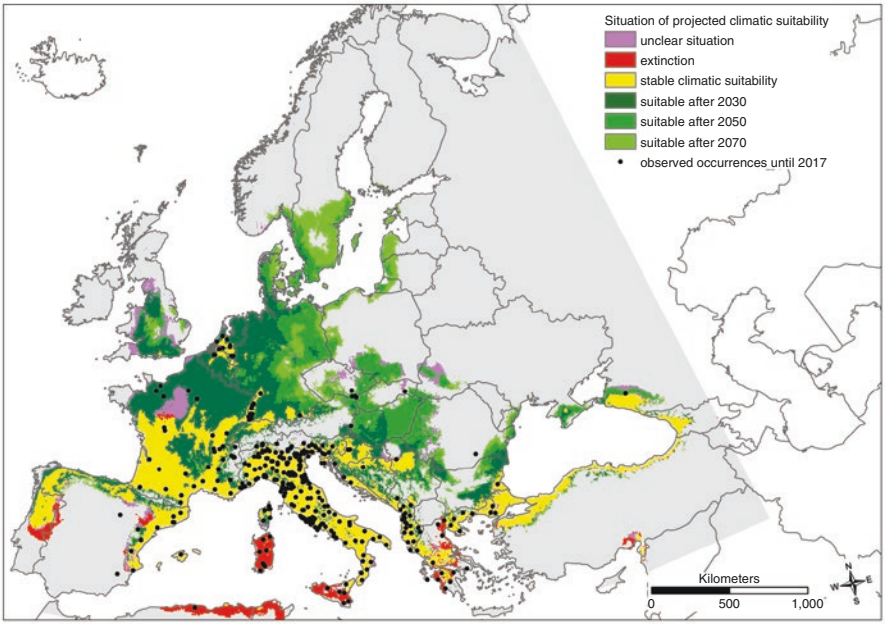

Fig. 10.2 Projected climatic suitability for *Aedes albopictus* in Europe under current and future climatic conditions (RCP 4.5). Observed occurrence records are displayed as black dots. Red indicates areas with projected climatic suitability under current climatic conditions but climatically not suited under future conditions (for all considered future time periods). Yellow indicates areas with climatic suitability continuously projected for all considered time periods (current, 2030, 2050 and 2070). Green indicates areas projected to be climatically suitable only under future climatic conditions (in dark green after 2030, in middle green after 2050 and in light green after 2070). Areas displayed in purple show areas with an unclear situation (i.e. projected suitability for some of the considered time periods but no clear trend, e.g. suitable under current climatic conditions, unsuitable in 2030 and 2050 but suitable in 2070)

Ecological niche modelling estimates the climatic suitability of a location/area for a species but does not assess whether the species is able to reach that area. The distributional patterns of species are driven by environmental conditions (abiotic factors) but also by biotic interactions (biotic factors) and mobility (e.g. migration ability, barriers to dispersal, Soberón 2010). As *Aedes albopictus* is considered to be a strong competitor, disregarding biotic interactions in ecological niche models of this species is only of minor importance. However, neglecting the dispersal ability is generally considered a major problem in correlative species' distribution modelling approaches (Thuiller et al. 2008). The spread of a species can take place without further aids (intrinsic migration) or by means of, e.g. human-mediated transport vectors (e.g. trade of used tyres). The modelling results presented in Fig. 10.2 only account for climatic suitability for *Ae. albopictus* and disregard species' mobility. Studies should therefore also take migration ability into account in order to project the potential future distribution of the species more accurately. Here, in a first attempt, intrinsic migration ability was considered.

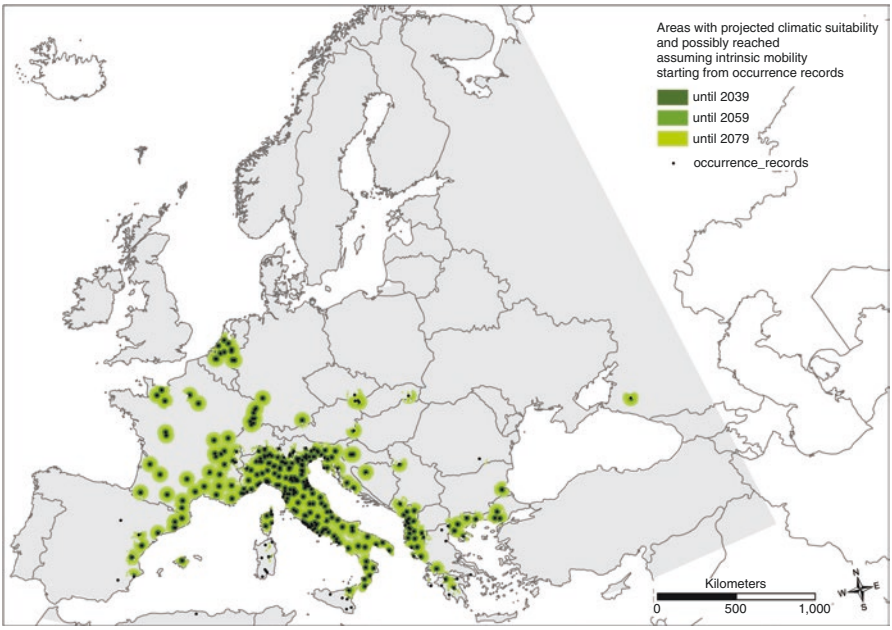

Fig. 10.3 Areas of projected climatic suitability accounting for dispersal ability (intrinsic migration ability). Green areas indicate potential range expansions for *Aedes albopictus* in Europe accounting for climatic suitability (RCP 4.5) and dispersal ability assuming an intrinsic migration rate of 800 m per year: dark green area is projected to be climatically suitable and possible reached until 2039, middle green area is projected to be climatically suitable and possible reached until 2059, and light green area is projected to be climatically suitable and possible reached until 2079 according to the niche modelling results based on the ensemble forecasting with six climatic variables, the RCP 4.5 and assumed migration rates of 800 m per year

Although *Ae. albopictus* is assumed to travel only less than 200 m in distance during their lifetime (e.g. Bonnet and Worcester 1946; Mori 1979), recapture experiments have shown an ability to cover distances up to 800 m (Niebylski and Craig 1994; Honório et al. 2003; Bellini et al. 2010). For the realization of range shifts, rare long-distance dispersal events are highly important (Nathan 2006). In the modelling approach applied here, the assumed migration rate was set to 800 m per year and one generation per year. A buffer analysis was carried out in ArcGIS (version 10.3, www.esri.com) in order to identify areas potentially reached until a certain time period and then intersected by the areas modelled to provide suitable climatic conditions within the respective time period. Based on the occurrence records of *Ae. albopictus* in Europe, areas that can be possibly reached within different time periods (until 2039, 2059, 2079) are displayed in different shades of green in Fig. 10.3.

As already pointed out, apart from climate change and socio-demographic evolution, the increasing international travel and transport of goods are factors that might greatly facilitate the introduction of vectors. This in turn constitutes a fundamental step in the process of a possible establishment and invasion. Thus, the results in

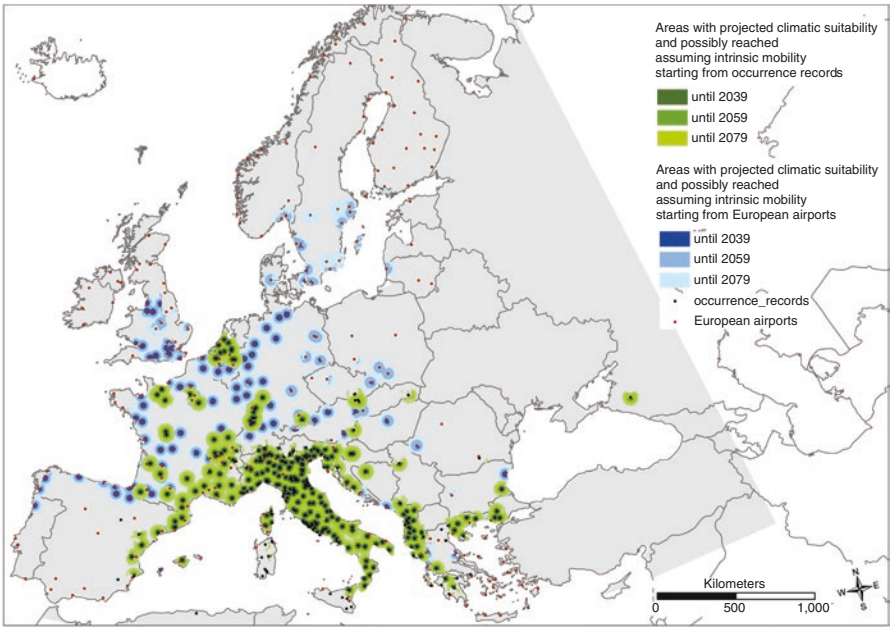

Fig. 10.4 Areas of projected climatic suitability accounting for dispersal ability (intrinsic migration ability and air travel). Green and blue areas indicate potential range expansions for *Aedes albopictus* in Europe, starting from occurrence records (green) or airports (blue): dark green/blue area is projected to be climatically suitable and possibly reached until 2039, middle green/blue area is projected to be climatically suitable and possible reached until 2059, and light green/blue area is projected to be climatically suitable and possible reached until 2079. The results are based on the ensemble forecasting with six climatic variables, the RCP 4.5 and an assumed migration rates of 800 m per year. European airports: Esri_Belux_Content feature service

Fig. 10.3 might still underestimate the potential future range of *Ae. albopictus* in Europe as they omit potentially important entry points of the mosquito species. In a second step, air travel was therefore taken into account as a possible way of dispersal. The area possibly reached by *Ae. albopictus* individuals assuming an intrinsic migration rate of 800 m per year taking airports as a potential source of newly arriving individuals is displayed in different shades of blue in Fig. 10.4.

Regarding the future threat of disease outbreaks caused by mosquito-borne pathogens in Europe, the potential future spread of vector-competent species (non-native but also native) is of major concern. Niche modelling is a very valuable approach to identify areas that are climatically suitable. However, it is very unlikely that all climatically suited areas will be occupied by the respective species within the next years or decades. Range expansion or range shifts take time. Due to limitations in the species' inherent ability to migrate, but also due to topographical dispersal barriers like mountains or oceans, the area for which climatic suitability is modelled always constitutes an overestimation of the potential future distribution of the species. Accounting for dispersal would therefore improve the modelling results.

Here, two rather simplified approaches were chosen to incorporate dispersal into the modelling framework, but results are subject to uncertainties and likely underestimate the potential future distribution of *Ae. albopictus* in Europe for several reasons. Firstly, assumptions made about intrinsic migration ability were only approximate. For example, other migration paths, especially human-mediated dispersal, may be very important for *Ae. albopictus*, but were not considered here. Those could be the indirect transport off eggs via trade of used tyres or lucky bamboo or mosquito individuals being trapped in cars of tourists when leaving their places of vacation (e.g. Roiz et al. 2011). To account for these different migration paths, important motorway routes (especially motorway restaurants) and railways (especially railway stations) should be also included in future modelling approaches. Secondly, occurrence data of species rarely does contain all locations of a species' presence, which could lead to underestimations of potential future distributions. The approaches including dispersal assumptions (Figs. 10.3 and 10.4) would allow more accurate predictions. However, *Aedes albopictus* is considered to be the fastest-expanding invasive species (Bonizzoni et al. 2013; Baldacchino et al. 2017), so the full dispersal assumption (i.e. assuming no propagation restriction, Fig. 10.2) might be worth to be considered, in order to recognize and prevent a further spread early on.

10.6 Risk Assessment and Conclusion

Europe has to face the (re-)emergence of different mosquito vectors and their associated pathogens (Osório et al. 2014; Kampen et al. 2015). Examples are the recurrence of the West Nile virus and the Usutu virus in recent years (Calzolari 2016) or the occasional indigenous epidemics in Italy in 2007 or France in 2010 (Tomasello and Schlagenhauf 2013; Cramer 2014). The (re-)emergence of vector-borne diseases is strongly related to the occurrence of competent vector species (Gratz 1999; Beerntsen et al. 2000; Ready 2010; Cramer 2014). Changes in climatic conditions, especially increasing temperatures, and changes in seasonal precipitation patterns are important key factors influencing the establishment and range expansion of vectors and pathogens (Akin and Martens 2014; Semenza et al. 2012; Cunze et al. 2016b), whereas non-climatic factors play an important role for the introduction of disease vectors and pathogens but also for the severity of the disease outbreaks (Beugnet and Chalvet-Monfray 2013).

Other risk-associated factors for disease outbreaks than those outlined in the above sections include the increase in population density, development of outdoor activities or a different access to health care (Semenza and Menne 2009; Beugnet and Chalvet-Monfray 2013; Akin and Martens 2014; Gould et al. 2017). Factors like mass migrations as a consequence of armed conflicts and war or illegal immigration are drivers for disease dispersal (Calzolari 2016). Another factor is the

change in land use and the increasing urbanization. Urban areas could have a positive effect on the breeding success and therefore the survival and activity of the vector species (Medlock and Leach 2015; Dzidová et al. 2016), e.g. some *Aedes* species have adapted to urban environments where they use small man-made containers for breeding and live inside houses (Gould et al. 2017).

Vector species can have different transmitting capacities, and even closely related species can differ in their vector competence (Becker 2008; Calzolari 2016). Moreover, the emergence and spread of a disease-causing agent (pathogen) can benefit from reservoir hosts, e.g. migratory birds. Reservoir hosts are able to maintain and sometimes even accumulate the pathogenic agent; they are long-living organisms in which pathogens can proceed with their development. Migratory birds are common reservoir hosts for many vector-borne diseases (Bairlein and Metzger 2014). They do not only provide suitable conditions for the development of the pathogen but also facilitate the spread of pathogens into new areas over large distances through their migration (Calzolari 2016). Host preference of different mosquito vectors additionally influences their disease-transmitting capacity (Calzolari 2016). Whether they are specialized to feed on only one specific host or are generalist feeders affects which type of diseases are transmitted.

While autochthonous cases of malaria, dengue fever and West Nile fever are the first evidence for the reintroduction of the disease-associated pathogens into Europe, the risk of large epidemics seems low. Nevertheless, preventive monitoring and surveillance programmes prove to be valuable and should be continued. In addition to the direct health impact, infections with mosquito-borne diseases are also an indirect health problem due to the lack of blood transfusions and organ donations for not infected people, the enormous costs for treatment and possible late effects of infections (Calzolari 2016). Facing the threat of future disease outbreaks, an early detection system is needed. As a first step, different European countries have started mosquito monitoring and surveillance programmes in order to detect invasive mosquitoes as well as pathogens and disease outbreaks at an early stage (Engler et al. 2013; Kampen et al. 2015). Unfortunately, effective surveillance is currently challenging due to the limitation of resources and methodological difficulties in mosquito identification and rapid pathogen detection (Engler et al. 2013). An integration of the most recent technical developments and a subsequent adjustment of monitoring and surveillance programmes are urgently needed as mosquitoes and their associated pathogens in Europe will continue to spread at a faster pace than ever before (Gould et al. 2017).

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Chapter 11
Mosquito-Borne Diseases: Prevention Is the Cure for Dengue, Chikungunya and Zika Viruses

Devi Shankar Suman, Kshitij Chandel, Ary Faraji, Randy Gaugler, and Kailash Chandra

Abstract Arboviruses diseases, especially dengue, chikungunya and Zika are responsible for millions of human sickness and a significant number of deaths worldwide. There is no effective treatment and cure available for these arboviral diseases. These diseases are transmitted mainly by *Aedes* mosquitoes such as *Aedes aegypti* and *Aedes albopictus.* They are highly anthropophilic, container-inhabiting and diurnal mosquitoes surviving in peridomestic habitats. The management of dengue, chikungunya and Zika diseases mainly depends on vector management, but the habit and habitats of these mosquitoes make their management difficult. The present chapter mainly has three major sections: (1) mosquito, viruses, interrelationships and disease treatment, (2) vector management and (3) novel approaches for vector management and disease transmission inhibition. The first aspect includes the relationship of mosquitoes with arboviruses, vector competency and the treatments of arbovirus diseases involving vaccine developments with future aspects. The next section deals with mosquito biology, surveillance and control strategies for eggs, larvae, pupae and adults stages using the conventional and advanced methods to develop an integrated approach for effective vector management. This section also elaborates the environmental management, biological and insecticidal controls of different stages of mosquitoes. The last segment of the chapter discusses the efficacy and applicability of novel technologies for multiple stages of *Aedes* mosquito control which includes autodissemination technology, *Wolbachia*-based cytoplasmic incompatibility, sterile insect techniques and the release of insects carrying a dominant lethal gene (RIDL) that can be a potential tool in the future to restrict

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disease transmission. The human behaviour alteration to avoid mosquito biting and methods applicable for personal protection has been discussed in the chapter. Based on the discussion, the prevention of the transmission of dengue, chikungunya and Zika is the only cure in the present scenario.

Keywords Arboviruses · Vector-borne disease management · *Aedes* mosquitoes · Surveillance methods · Vector control · Novel mosquito control approaches · Autodissemination · RIDL

11.1 Introduction

Mosquito-borne diseases are threats even in the twenty-first century and thus have become a focal point for the researchers and the world community (WHO 2014). Vector-borne diseases alone contribute 17% of all the infectious diseases combined are responsible for >1 million deaths worldwide. The World Health Organization (WHO) reports that more than 2.5 billion people in over 100 countries are at risks of dengue infection alone (WHO 2014). Several other virus species were reported from the mosquitoes back in the twentieth century and estimated 100 of those virus species were able to cause infection (Gould et al. 2017). The emergence of Zika virus in Americas in recent years has caused thousands of microcephaly cases in the American continent (WHO 2017). In recent years, a great number of dengue and chikungunya viruses occurred in many Asian countries (WHO 2009). The danger of mosquito-borne pathogens is real with dengue, chikungunya and Zika viruses which are being transmitted by mosquito *Aedes aegypti* and *Aedes albopictus.*

There is no effective treatment or vaccine available for dengue, chikungunya and Zika viruses. Thus far the vector control is the most reliable means to manage the disease transmission and prevention (WHO 2009). The ecological changes facilitated the spread of zoonotic and sylvatic diseases by the interactions of arboviruses with vector insects as well as their compatibility (Liang et al. 2015). The vector status is determined by the combined effect of physiological and ecological factors of vector, host, pathogen and environment (Mitchell 1983).

These mosquitoes are widespread around the world. In recent years, *Ae. albopictus* has been able to establish its population in the USA and European countries because of its adaptability to adverse cold environmental conditions (Suman et al. 2015). Both *Ae. aegypti* and *Ae. albopictus* are diurnal and anthropophilic and can survive in the periphery of human residence and inhabit water-holding containers for oviposition and larval development (Hawley 1988). These conditions facilitate them to find the host and habitats in close range making them more threatening to human populations.

By considering, the biology, host-seeking and oviposition behaviours of *Aedes* mosquitoes species, new tools and techniques have been developed for the surveillance and treatment of mosquitoes such as BG-Sentinel traps. Because the methods used for nocturnal *Anopheles* and *Culex* mosquitoes were im- or partially efficient against the *Ae. aegypti* and *Ae. albopictus*, surveillance is the backbone of any control programme. Various ovitraps and adult traps have been used for effective

surveillance of egg and adult stages, respectively. These traps have been supplemented with infusions or lure to enhance the efficacy (Ritchie et al. 2014; Trexler et al. 1998). Similarly, the applications of recommended insecticides have been evaluated by several laboratories to treat the larval habitats and adult populations by utilizing a hand-held, backpack and truck-mounted sprayer to reduce the manpower and make it more cost-effective (Farajollahi et al. 2012; Suman et al. 2014; Williams et al. 2014). In addition, there are several other methods that have been found effective to reduce mosquito population effectively.

Elimination of *Ae. aegypti* and *Ae. albopictus* mosquitoes is the most difficult task (Fonseca et al. 2013). Studies have shown that cryptic habitats of these mosquitoes are immune to most effective and powerful machines used for spraying larvicides and support the population resurgence (Unlu et al. 2013; Chandel et al. 2016). Several devices and methods have been used with pyriproxyfen, juvenile hormone analogue, which have shown promising results (Devine et al. 2009; Gaugler et al. 2012; Caputo et al. 2015; Suman et al. 2017). To enhance the penetration of larval habitats, we and others have developed novel methods of autodissemination. For adults, nighttime ULV spray and attractive toxic sugar bait applications have been found to be effective to reduce populations (Muller et al. 2010a; Farajollahi et al. 2012; Qualls et al. 2014). The recent developments in molecular sciences, genetics and microbiology techniques have also been provided new avenues to curtail the mosquito population and block the disease transmission (Xi et al. 2005b; Coon et al. 2016; Van Den Hurk et al. 2012).

The anthropophilic nature of *Aedes* mosquitoes has a direct impact on human activities (Worobey et al. 2013). Therefore, protection by using repellents and insecticide-treated clothes have become considerable important to avoid mosquito biting. An effective repellent can protect a person from mosquito biting up to 8 h, whereas treated clothes were able to reduce landing and biting rates of mosquitoes (Schreck and McGovern 1989; Orsborne et al. 2016). This shows the possibility of the reduction of the arboviral case if used properly. This is a public concern; hence, the education and participation of residents and another volunteer can help reduce mosquito bite and possibly reduce mosquito populations.

In this chapter, we have discussed the biology, surveillance and control measures of different life stages of *Ae. aegypti* and *Ae. albopictus* mosquitoes. The development of novel vector control techniques for eco-friendly controls and personal protection measure to minimize the mosquito bite may be key to arresting arbovirus disease transmission.

11.2 Mosquito Viruses and Vector Competency

Vector competency is the vector's susceptibility to oral infection and capability to multiply or propagate and further successful transmit the diseases parasite in a healthy individual. Various extrinsic and intrinsic factors contribute to the competence of mosquito vectors for various viral agents. Extrinsic factors such as temperature, humidity

and nutrition have been shown to have a direct impact on mosquito fitness, survival and parasite development (Agarwal et al. 2017). Temperature is one of the key factors that affect the biology of mosquito vector and thereby influence vector life cycle, population density, adult survival and susceptibility to a viral pathogen. Studies have shown that the higher temperature fluctuation between day and night can reduce incubation period of dengue virus (Carrington et al. 2013). In contrast, a spread of the virus to different body parts of mosquito was greatly reduced, when immatures were reared in cold water (Alto and Bettinardi 2013). Humidity and precipitation are also responsible for mosquito population buildup. Precipitation results in an exponential increase in larval breeding habitats and higher humidity provide a conducive environment for the adult mosquito to survive. Changes in environmental temperature and humidity influence the extrinsic incubation period (EIP); it's the incubation period from the time when mosquito acquires a viremic blood meal to the time when a mosquito is ready to transmit a virus to a healthy individual. Increase in temperature and humidity leads to the shorting of EIP and increased blood-feeding frequency (Tseng et al. 2009). A positive correlation has been observed in rainfall and dengue incidence in Thailand and Latin America (Indaratna et al. 1998; Goncalves Neto and Rebelo 2004). Another important extrinsic factor is quality and quantity of the food, which has a direct relation to mosquito fitness and survival. For instance, scarcity of food during larval stage has been reported to delay in malaria parasite development in adult *Anopheles stephensi* mosquito (Shapiro et al. 2016).

There are growing agreements that midgut physiology, microbiota, mosquito immune response and genetic makeup are the intrinsic factors which influence vector competence (Agarwal et al. 2017). In virus transmission cycle, mosquito gut plays an important role and is a site where the virus first encounters the mosquito environment. Various physiological and biochemical changes take place in mosquito gut after blood feeding. Reversible functional changes induced in mitochondria of *Ae. aegypti* females after blood feeding, which leads to a reduction in oxygen consumption and hydrogen peroxide generation during early and mid-phase of blood digestion which reaches its higher level during the late phase (Goncalves et al. 2009). At the molecular level, transcription factors, ion-binding proteins and other metabolic proteins are upregulated in *Ae. aegypti* females during flavivirus infection, and genes responsible for proteases and pupal cuticle proteins are downregulated (Colpitts et al. 2011). Mosquito ubiquitin protein Ub3881 is highly downregulated during dengue virus infection in *Ae. aegypti* females (Colpitts et al. 2011). Ubiquitin is involved in degradation of dengue virus envelop protein leads to reduced number of virus released from an infected cell (Agarwal et al. 2017). Another protein, C-type lectins play a significant role in the establishment of flavivirus infection in mosquito vectors (Perera-Lecoin et al. 2014; Prommalikit and Thisyakorn 2015). Knockdown of mosGCTL-1 gene or administration of antimosGCTL-1 antibody has been found to reduce infection of WNV in *Aedes* and *Culex* mosquito (Cheng et al. 2010). *Aedes aegypti* midgut protein carboxypeptidase B1 (CPB1) also interacts with DENV-2 envelope protein, and in the presence of CPB1, virus released from infected cells could not complete their protein assembly, thus reducing the chances of salivary gland colonization (Tham et al. 2014).

Mosquito gut provides a conducive environment for the microorganism to flourish and, a wide variety of bacterial species have been isolated and identified from mosquito gut (Chandel et al. 2013; Yadav et al. 2015; Muturi et al. 2017). These microorganisms can affect vector competence of mosquito either by inducing host immune system or by directly interacting with virus agent (Cirimotich et al. 2011). In a study, the absence of midgut bacteria that has resulted in enhanced vector competence of mosquito vector such as *Anopheles* mosquitoes that was reared on an antibiotic-supplemented diet (to reduce midgut bacteria) has shown increased susceptibility to *Plasmodium falciparum* (Dong et al. 2009). A similar observation in *Ae. aegypti* mosquitoes has shown an increased level of dengue infection in females exposed to antibiotics (Xi et al. 2008). Some studies have also shown that the presence of some bacteria species can enhance vector competence such as susceptibility to *Ae. aegypti* to dengue and chikungunya virus is greatly increased in presence of *Serratia odorifera* in mosquito gut (Apte-Deshpande et al. 2012, 2014). On the other hand, some bacterial species have shown a deleterious impact on vector competence of mosquito vector. *Plasmodium falciparum* invasion in midgut epithelium is greatly reduced in presence of *Enterobacter sp.* in *Anopheles* mosquito (Cirimotich et al. 2011).

Mosquito exerts an innate immune response on exposure to the infectious parasite. This immune response is the first line of defence against any foreign infection including arboviruses. The JAK-STAT, Toll signalling and RNAi pathways are major immune response elicited by arbovirus infection in mosquito vector (Colpitts et al. 2011; Renteria et al. 2015). Mosquito immune response reduces the viral pathogenesis during infection. Upon dengue virus infection, *Ae. aegypti* females induce a Toll-mediated immune response to suppress the infection (Xi et al. 2008). *Aedes aegypti* mosquito also relies on RNAi-mediated immune response for silencing viral gene expression upon dengue and other arbovirus infections (Vargas et al. 2009). Arbovirus has to overcome these defence mechanisms to successfully complete transmission cycle in mosquito host.

Another important intrinsic factor is the genetic makeup of mosquito vectors and viruses. The genetically different population of *Ae. albopictus* mosquito has shown differential vectorial competence for CHIKV oral infection (Tesh et al. 1976). The study conducted by Mercado-Curiel et al. (2008) has suggested a role of dengue receptor R67/R64 proteins (67 kDa protein) as a vector competence marker in *Ae. aegypti* mosquito for dengue virus (Mercado-Curiel et al. 2008). Similarly, *Ae. aegypti* populations, differing in isozyme profiling, have shown variable susceptibility to yellow fever virus (Tabachnick et al. 1985). Different *Ae. albopictus* geographic strains have shown differential susceptibility to CHIV oral infection (Tesh et al. 1976).

11.3 Arbovirus Diseases and Their Treatments

There are approximately 100 arboviruses that are known to cause diseases in human hosts, but the severity of disease ranges from asymptomatic infection to life-threatening encephalitis or haemorrhagic fever. The most common

arboviruses transmitted by mosquitoes are dengue virus (DENV), yellow fever virus(YFV), chikungunya virus (CHIKV), Rift Valley fever virus (RVFV), West Nile virus (WNV) and Japanese encephalitic virus (JEV) (WHO 2014). A recent outbreak of Zika virus has also gained the attention of public health research community because of its involvement in the development of microcephaly in newborn babies.

Dengue is one of the most rapidly expanding vector-borne disease. The causative agent of dengue is dengue virus (serotype DENV1, DENV2, DENV3 and DENV4) that is more dominant in tropical and subtropical regions. Dengue is endemic in over 100 countries, and almost 40% of world population is at risk of dengue infection; approximately 100 million dengue cases are reported every year (WHO 2014). The World Health Organization reports approximately 30-fold increase in dengue incident globally over the past 50 years (WHO 2009). Dengue virus infection can be mild illness involving fever and body ache to the most severe and life-threatening dengue shock syndrome and dengue haemorrhagic fever (WHO 2014).

Another arbovirus transmitted by *Aedes* mosquito is chikungunya virus (CHIKV). Earlier chikungunya virus was not considered as a major arboviral disease until it caused a global epidemic in 2005–2006. The epidemic was the result of and point mutational replacement of alanine to valine in E1 glycoprotein gene in Reunion Island strain, which led loss of cholesterol dependence for virus life cycle (Tsetsarkin et al. 2007). The mutated CHIKV strain was more adaptive for *Ae. albopictus* mosquito without losing its infectivity to *Ae. aegypti* females. Interestingly, *Ae. albopictus* was not an efficient vector, and *Ae. aegypti* was the major vector (Vazeille et al. 2007). This change in vector preference has led to its global epidemic reported from around 40 countries (Staples et al. 2009). In India alone, around 1.3 million CHIKV infections were reported during 2006 outbreak (WHO 2007). Chikungunya virus infection is mainly characterized by acute and chronic articular manifestations with severe polyarthralgia and fever. Although chikungunya infection is not life-threatening, severe joint pain in fingers, knees, ankles, wrists, elbows and other proximal joints can last for months and then eventually leads to loss to the active participation of individuals in society. It is estimated that there was a loss of 25588 DALYs (disability-adjusted life years) during 2006 CHIKV outbreak in India (Krishnamoorthy et al. 2009). In some cases, like in immune-compromised patients, CHIKV infection may be life-threatening because of the occurrence of encephalopathy, encephalitis and in rare cases multiorgan failure (WHO 2014).

Until now there is no effective antiviral treatment available to treat arboviruses. Symptomatic or supportive care and management of medical complications are the only mean to overcome arbovirus infections. Moreover, there is no FDA-approved antiviral treatment available for dengue virus; the only treatment that is available relies on maintenance of body fluid volume and palliative care. The treatment of acute chikungunya infection is comprised of administration of paracetamol or acetaminophen for fever and anti-inflammatory to relieve joint pain (WHO 2014).

11.4 Future of Vaccine Development: Probability of Success vs Failure

A vaccine can be an effective strategy to control arbovirus infections. Although vaccines are available for yellow fever and Japanese encephalitis viruses, there is no specific vaccine available for the treatment of other arboviruses. Yellow fever vaccine is a live-attenuated virus from the 17D lineage; a single dose of this vaccine can provide protection from infection up to 10 years. However, to maintain a lifelong protection from yellow fever infection in the endemic area, a booster dose after every 10 years is required (Coutinho-Abreu and Ramalho-Ortigao 2010). The development of a yellow fever vaccine has protected millions of human population from potentially fatal infection. In case of JE virus, four different types of vaccines are available, mouse brain-derived killed vaccine, cell culture-derived killed vaccine, cell culture-derived attenuated vaccine and genetically engineered live-attenuated chimeric vaccine (Alphey 2014). The first vaccine developed against JEV is mouse brain-derived vaccine which was first used in Japan in 1954 (Hoke et al. 1988), and later this vaccine was replaced by cell culture-based live-attenuated and killed vaccine. Genetically engineered live-attenuated chimeric vaccines (IMOJEV, JE-CV and THAIJEV) are commercially available in Australia and Thailand (Hoke et al. 1988; Halstead and Thomas 2011). A single dose of this vaccine has provided nearly complete (99%) seroconversion in adults. In Phase II trial, this vaccine provided protection against JEV infection for approximately 5 years in 87% of the vaccine recipients (Nasveld et al. 2010).

In last decades, great efforts have been made to develop a vaccine against dengue virus. Although there are few vaccine candidates available in various stages of development (Schwartza et al. 2015), still, there are not a single commercially available vaccines against dengue virus. It has been reported that primary infection with one type of dengue serotype can provide lifelong protection against that particular serotype (homotypic immunity) (Halstead 2013) and cross-protection for other serotypes for around 2 years (Reich et al. 2013). However, after that period, the person is still susceptible to dengue infection by other serotypes, and in some cases, it increases the severity of subsequent infection (Burke et al. 1988). This homotypic protection against dengue virus serotype is one of the important factors to be considered while developing an effective vaccine against dengue virus. The first dengue vaccine, which is licensed for phase 3 clinical trials, is Dengvaxia® (CYD-TDV) developed by Sanofi Pasteur (Scott 2016; Vannicea et al. 2016). It is a tetravalent, live attenuated containing four recombinant yellow fever 17D strain expressing *prM* and E protein from each dengue serotype. Dengvaxia® has been evaluated in two phase 3 clinical trials known as CYD 14 and CYD 15. CYD 14 trial was conducted in five dengue-endemic Asian countries (Indonesia, Malaysia, the Philippines, Thailand and Vietnam) (Capeding et al. 2014), whereas CYD 15 trial was conducted in five dengue-endemic Latin American countries, i.e. Brazil, Colombia, Honduras, Mexico and Puerto Rico (Villar et al. 2014). The protective efficacy of Dengvaxia® for dengue infection was estimated to range from 50.2 to 76.6% for individuals from differ-

ent age groups and serotypes (Villar et al. 2015). In silico modelling has suggested that the introduction of Dengvaxia® vaccine at early adolescence stage could result in the reduction in dengue hospitalizations by 10–30% over time (Vannicea et al. 2016).

In addition to Dengvaxia®, other potential dengue vaccine candidates are in the process of development or are in clinical trials (Schwartza et al. 2015; Vannicea et al. 2016). One potential vaccine candidate developed by National Institute of Health, USA, is a live-attenuated (recombinant) vaccine. TV003 and TV005 which are identical vaccine candidates except for the dose level of the DEN2 component were based on wild-type strains mutated to attenuate the virus [28]. Another potential vaccine candidate Denvax is a tetravalent vaccine using DENV-2 and recombinant prM and E proteins of the rest three serotypes. Both vaccine candidates have shown promising results and now are in phase 2 trials (Durbin et al. 2013; Osorio et al. 2014; Kirkpatrick et al. 2015).

Similar to dengue virus, there is no vaccine available against CHIKV infection. However, prospects look good because there are more than 15 vaccine candidates against CHIKV infection that are in various stages of development. These vaccine candidates are based on various platforms such as inactivated, live-attenuated, chimeric, subunit protein and DNA-based vaccine (Smalleya et al. 2016). Although research is yielding important insights, most of the candidate's vaccine are in preclinical stages, and only a few have reached to phase 1 clinical trials (Smalleya et al. 2016).

Availability of an effective vaccine can become a powerful tool to fight mosquito-borne viral diseases; however, a details risk assessment in geographically diverse population has to be performed prior to approval for public use.

The conventional vaccine can only protect individuals from infection, but mosquito carrying arbovirus can transmit the virus to unvaccinated individuals. A vaccine which can prevent or block transmission cycle of pathogen inside mosquito vector is an alternate approach to stop arbovirus transmission in humans (Matuschewski and Mueller 2007). Research has been conducted to develop broad-spectrum transmission blocking vaccine (TBV) (Vargas et al. 2009). The difference between conventional and TBV is their mode of action; TBV prevents infection in mosquito vector in place of its human or animal hosts and blocks the further transmission in the mosquito (Coutinho-Abreu and Ramalho-Ortigao 2010). A potential protein candidate for a TBV against DENV infection is the putative cysteine-rich venom protein 379 (CRVP379) important for DENV infection; silencing CRVP379 resulted in reduced DENV infection in *Ae. aegypti* mosquitoes. Antibodies against CRVP379 could bound the native CRVP379 protein in mosquito gut, which eventually resulted in transmission disruption of dengue virus (Renteria et al. 2015). Another TBV molecule identified protein Pfs25 is able to prevent the acquisition of malaria from infected mice by female mosquitoes (Farrance et al. 2011). Similarly, mice vaccinated with Serpin-2 protein have prevented *Plasmodium berghei* acquisition in *Anopheles* mosquitoes (Williams et al. 2013).

TBVs have the potential to decrease arbovirus load in the wild mosquito population, which will also increase herd immunity and can be an effective tool to fight arbovirus infection. Available data has suggested that a single TBV vaccine may develop, which can provide protection from multiple viral agents.

11.5 Vector Control to Minimize the Risk of Disease Transmission

Arboviral and vector-borne disease transmissions are directly proportional to the frequency of mosquitoes bites (Mitchell 1983). Control check on the explosion of mosquito populations has become a crucial factor in reducing human-mosquito contact and minimizing the risk of disease transmission (WHO 2009). Unfortunately, there is no single shot for the management of *Aedes* mosquitoes; different strategies are applied for their population control (Faraji and Unlu 2016). These mosquitoes have highly specialized life stages that support their survival in adverse conditions and in a variety of habitats for their development (Hawley 1988). To keep the mosquito population below significant level is a great challenge because it is hard to find the target area and suitable application methods (Fonseca et al. 2013). The World Health Organization (2014) has promoted integrated vector management (IVM) as a strategic approach including the dengue vector control (WHO 2014). IVM is defined as "a rational decision making process for the optimal use of resources for vector control" which considers five key elements in vector management: (1) advocacy, social mobilization and legislation, (2) collaboration within the health sector and with other sectors, (3) integrated approach to disease control, (4) evidence-based decision-making and (5) capacity-building (WHO 2009). The third, integrated approach to disease control using vector management is generally categorized in two types of methods: (1) conventional method and (2) advanced methods. Figure 11.1 shows an overview of different methods of surveillance and control strategies for the management of *Ae. aegypti* and *Ae. albopictus* mosquitoes.

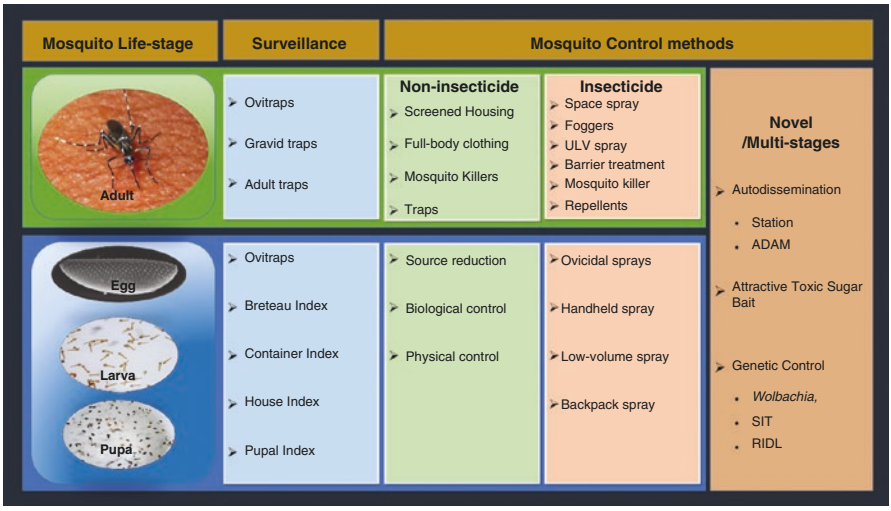

Fig. 11.1 Overview on surveillance and control strategies for the management of *Aedes aegypti* and *Aedes albopictus* mosquitoes

11.5.1 Conventional Methods

It involves tools based on environmental management, biological control agents and chemical insecticides for the vector management. These methods are well-established and have been used worldwide. The consideration of vector control is based on disease impact, epidemiology and infested areas.

11.5.2 Advanced Methods

These are newly emerging ideas, proof of concepts and methods at the early phase of their development and have been field evaluated in small scale but not have been accepted or evaluated at larger scales. The aim of the advanced technology is to provide eco-friendly, cost-effective and stable long-term solution for mosquito control. These techniques are based on the exploitation of their mating, oviposition or host-seeking behaviours as well as genetic methods, for example, autodissemination technology, SIT, RIDL, lethal ovitraps, attractive toxic sugar baits, etc.

In the following section, the biology of each life stage, their surveillance and available methods of vector control have been discussed.

11.5.3 Eggs Biology, Surveillance and Control

Mosquito eggs are an important stage in mosquito life history and one of the deciding factors for the successful establishment in a specific area (Hawley 1988). Egg encloses in multiple chorionic layers that synthesize during egg and embryonic development. These layers provide rigidity and mechanical support to the eggshell and protect the embryo from different forces such as shaking and collapsing due to drying (Clements 1992). *Aedes aegypti* and *Ae. albopictus* eggs highly evolved for the semiurban and urban ecology inhabiting in containers inside the house and outside in backyards. *Aedes* eggs are highly tolerant to desiccation and are more resistant to collapse than the *Culex* eggs (Sota and Mogi 1992; Suman et al. 2014). Egg features and oviposition biology of *Ae. aegypti* and *Ae. albopictus* complicated their management. These mosquitoes prefer cryptic habitats and are adapted to live in a variety of multiple containers to avoid risks utilizing skip-oviposition behaviour (Sihuincha et al. 2005; Davis et al. 2016; Wang et al. 2014). Females deposit their eggs individually on the inside wall of the container just above the water by sticking mechanism that supports egg persistence for a long time even after the container water is removed or containers dried out during the summer. In contrast, the eggs of *Culex* and *Anopheles* float freely on the water surface. In *Ae. aegypti* and *Ae. albopictus*, eggs can survive for several months under optimal weather conditions once they are embryonated, and eggs hatch when water accumulates in the rainy season.

Also, *Ae. albopictus* eggs have the tendency to go in diapause in temperate conditions to overcome the cold temperature. The diapause in gravid females is induced by short-day photoperiod and lower temperature to synthesize necessary reserves and cryo-preservative during embryonic development (Suman et al. 2015). In *Ae. albopictus*, diapause accomplishes in a fully embryonic developed egg and is ready to hatch in favourable conditions. Eggs backup the population resurgence in next season in infested areas.

11.5.3.1 Surveillance

An effective surveillance of eggs can provide significant time to prepare for proactive control measures because the development of adult population from egg takes almost 1–2 week time (Suman et al. 2017). Generally, ovitraps are simple cup-/tray-like designs that are supplemented with oviposition substrate and infusion water as an attractant for gravid females (Reiter et al. 1991; Trexler et al. 1998; Unlu et al. 2017). Ovitraps have been used by several workers to estimate egg populations of both *Ae. aegypti* and *Ae. albopictus* (Bellini et al. 1996; Abramides et al. 2011; Fonseca et al. 2013; Achee et al. 2015). These traps are user-friendly because of low maintenance, work without electricity and are cost-effective. Codeco et al. (2015) have suggested that the ovitraps can be a sensitive tool to detect the presence of container *Aedes* mosquitoes and assessing adult population dynamics. However, ovitraps may not be sensitive enough at the low population density of adults in comparison to adult traps (Fonseca et al. 2013). Moreover, if multiple containers inhabiting *Aedes* species exist in the same area, there are chances to get mixed eggs which need to be resolved by post-emergence identification of adult proportion to estimate accurate density (Farajollahi and Price 2013; Fonseca et al. 2013). By neglecting minor critiques on sensitivity, ovitraps can be considered an effective tool to detect the *Aedes* mosquito infestation by homeowners and mosquito control agencies.

11.5.3.2 Controls

Efforts have been made in search of effective and efficient ovicidal agents, yet insecticidal management of egg population has not been recommended for *Aedes* control. The susceptibility of eggs to different insecticides is reported to be significantly lower than their larval stages (Vasuki 1990; Suman et al. 2013). Suman et al. (2013) have found that the freshly laid *Ae. albopictus* eggs showed 80.6%, 42.9% and 35.8% mortality for 1 ppm of pyriproxyfen, azadirachtin and diflubenzuron, respectively, which was lower for *Ae. aegypti* eggs (47.3%, 15.7% and 25.5%, respectively).

Suman et al. (2013) also found low ovicidal efficacy for the mosquito management at recommended concentrations of insect growth regulators (Suman et al. 2013). Other studies by Vasuki (1990) and Su and Mulla (1998) also reported the similar efficacy *Culex quinquefasciatus* eggs. There is an interesting finding reported

by Suman et al. (2015) which showed that pyriproxyfen performs the dual functions; one induces the hatching of diapause eggs at low concentration, and other kills egg at the higher dosage. The bottleneck is the dosage required for an ovicidal activity, for *Aedes* mosquitoes are much higher than WHO-recommended concentrations of insecticide, and such dosages are not advisable to use for the control of mosquito control.

In future, ovicides may become an important tool in mosquito management because eggs help population resurgence as well to overcome the adverse conditions. There is an urgent need to expedite research on ovicides for the development of better formulations with higher penetration to eggshell and embryonic membranes to enhance the efficacy at recommended larvicidal concentrations of insecticides, as well as effective delivery methods that can treat the cryptic habitats where eggs are hidden (Chandel et al. 2016). The ovicidal activity can delay population buildup by depleting the reserve of the mosquito population.

11.5.4 Larval-Pupal Biology, Surveillance and Control

Larval stages are the second aquatic stage in mosquito life cycle and play a crucial role in maintaining mosquito population because of *Ae. aegypti* and *Ae. albopictus* larvae that cannot arrest the development after egg hatch. Larvae moult 1st to 4th instars before turning in to pupae. Both stages are active swimmers, but pupae do not feed contrary to larvae (Clements 1992). Pupae emerge into adults after final moult. All the moulting are controlled by insect hormones that provide checkpoints for mosquito management. The larval and pupal populations are important and early indicators for adult population's estimation rather than the eggs because visualization and estimation of egg density are not feasible in both the field condition and natural habitats. *Aedes albopictus* and *Ae. aegypti* are peridomestic and inhabit heterogeneous larval habitat ranging from natural to artificial containers including water pools in the backyard, buckets, tyres and catch basins (Russell et al. 1996; Unlu et al. 2013). The information of the larval habitats of any mosquito vector species is not only important for the surveillance but also the control point of view. The mosquito usually passes 7–10 days in these aquatic stages in the same habitat which provides a large window for larvicidal actions and controlling mosquito populations.

Both *Ae. albopictus* and *Ae. aegypti* mosquito species are skip ovipositor and generate numerous habitats of diverse types. These habitats can be open in nature or cryptic condition which is hard to locate mosquito control team or unable to be exposed to mosquito control tools (Unlu et al. 2013; Chandel et al. 2016). Larval habitat selection for the oviposition is governed by several factors that provide cues to gravid females. These oviposition cues can be colour and texture of the habitat surface, quality of water, types and quantity of organic components, the presence of conspecific larvae and disturbance (Trexler et al. 1998; Hoel et al. 2009).

11.5.4.1 Surveillance

To estimate the mosquito population density, several methods of surveillance have been developed for larvae and pupae of container-inhabiting *Ae. aegypti* and *Ae. albopictus*. But these mosquitoes breed in containers make repetitive sampling difficult. To overcome the problem, WHO has recommended sampling with presence and absence of positive or negative containers for mosquitoes (WHO 2009). The surveillance of *Aedes* mosquitoes is based on larval indices and has been also recommended by WHO to estimate the infestation of these mosquitoes in targeted areas (WHO 2009). These indices are Breteau index (BI), a number of positive containers per 100 houses inspected; house index (HI), the percentage of houses infected with larvae and/or pupae; and container index (CI), the percentage of water-holding containers infected with larvae or pupae. Recently, a pupal index, number of pupae per 100 houses, has also been developed that is more realistic and reliable than BI, HI and CI (Focks and Chadee 1997). Although there is no direct evidence or relationship between these indices and disease prevalence, all these indices can be used to identify the target area of prevention where more efforts are needed and resources are to be translocated.

11.5.4.2 Larval Control

The control of larval or pupal stages plays a significant role in mosquito population management. The World Health Organization (2009) provided clear-cut guidelines for management of this aquatic stage, which involves (1) environmental management, (2) biological control and (3) chemical control.

11.5.4.3 Environmental Management of Immature Stages

The environmental changes are an effective method to prevent container dwelling *Aedes* mosquito propagation. The World Health Organization (2009) has suggested three types of environmental management: (1) **environmental modification,** long-lasting physical transformation for habitat reduction; (2) **environmental manipulation,** temporary changes to vector habitats; and (3) **changes to human or behaviour,** to reduce human-vector contact.

The World Health Organization (2009) has categorized breeding habitats of both *Ae. aegypti* and *Ae. albopictus* with a list of methods for their effective management (WHO 2009). These habitats include common types of breeding waters, i.e. water storage tank or cistern, drums, buckets, flower vase, saucers of potted plants, roof gutter, animal water container, discarded food and drink containers, hollow fence pots, used tyres, discarded large appliances, tree holes, rock holes and many other containers. Several easy methods of the source reduction are recommended to sanitize larval breeding habitats or to make them mosquito-proof for future breeding sites such as weekly empty, clean or scrub, putting mosquito-proof covers, store

under roof to avoid rain catch water, modify design or repair for easy cleaning, filling of polystyrene beads, soil and sand, collection of recycling containers and dispose of, and puncturing or draining (WHO 2009).

The effectiveness of the source reduction to control the container-inhabiting *Aedes* mosquitoes has been shown in various studies (Hawley 1988). Daily source reduction in recreational areas in China has claimed 50% reduction in *Ae. albopictus* population for a couple of weeks (Zhou et al. 2009). Targeting the key larval habitats alone can reduce 15% population of *Ae. aegypti* pupae in Peru (Wong et al. 2012). It is also suggested that adding source reduction with another tool enhances its efficacy (Wheeler et al. 2009). Unlu et al. (2013) have shown 75% reduction of *Ae. albopictus* population in treatment sites vs untreated sites when the source reduction and ULV adulticide were combined (Unlu et al. 2013). However, source reduction is labour-intensive, time-consuming and expensive (Zhou et al. 2009; Rao 2010; Fonseca et al. 2013).

In some cases, source reduction is almost impossible when breeding habitats are numerous and not under reach to mosquito control team peoples such as locked houses and backyards, private properties, junkyards and tree holes. Moreover, gravid *Ae. aegypti* and *Ae. albopictus* females generate multiple breeding habitats by skip-oviposition behaviour (Sihuincha et al. 2005; Wang et al. 2014). These multiple and new breeding containers exacerbate the problem over time (Bartlett-Healy et al. 2011). *Aedes albopictus* oviposition behaviour may change to exploit new habitats or delayed egg hatch. This phenomenon is known as "bet-hedging" theory which suggests that parents stagger offspring emergence into vulnerable life history stages to avoid catastrophic reproductive failures (Venable 2007; Khatchikian et al. 2009). In other conditions, that make source reduction inefficient, for example, mosquito habitats are just not removable or owners do not allow to treat them such as bird baths, corrugated extension spouts, underground storage tanks, water humidifiers, drinking water overhead tanks, etc. Generally, mosquito control departments do not pose large teams to support door-to-door source reduction in the peridomestic environment as well as lack of motivation in homeowners to keep their surroundings larval habitat-free making source reduction tedious and logistically inoperable. This method may be more effective if it is merged into the traditional way of life and daily activities through the educational system, human activity centres and public gathering places.

11.5.4.4 Biological Control of Larvae

Environmental controls of *Ae. aegypti* and *Ae. albopictus* that provide limited efficacy are labour-intensive and hard to convince the public to use it. The biological control provides a natural way to suppress the mosquito population by utilizing parasites and predators against larval and pupal stages of aquatic mosquitos. The World Health Organization (2009) also recommends the use of biological control agents for the management of *Ae. aegypti* and *Ae. albopictus* mosquitoes.

There are several organisms known to be good biological control agents against mosquitoes (Huang et al. 2017). Biocontrols have been found to be the best suitable

method of control for *Anophelines* and *Culicine* vectors whose habitats are large permanent water bodies. For example, *An. culicifacies*, a rural malaria vector, breeds in ponds, puddles and riverine belt. Application of a mosquito fish, *Gambusia affinis* against this vector, has shown promising efficacy in reducing the mosquito population and malaria cases in India (Naucke et al. 2007). In another example, mermithid nematodes, *Romanomermis iyengari*, have been successfully used for the control of *Anopheles* mosquito larvae in Cuba and Mexico (Mijares et al. 1999; Santamarina Mijares and Perez Pacheco 1997). This nematode species is an effective candidate for the biological control of various mosquito vectors including *Ae. aegypti* (Paily and Balaraman 2000). Contrary to *Anopheles* and *Culex* habitats, the ephemeral habitats of container-inhabiting *Ae. aegypti* and *Ae. albopictus* mosquitoes may not be suitable for large size biological control agents such as larvivorous fish because the mosquito habitats are insufficient to satisfy the nutritional requirements of the pray or predators. This may also be difficult to use mermithid nematodes in *Aedes* habitats due to minimum drought tolerance of nematodes. Therefore, temporary nature of the water containers and frequently drying condition in summer may not support biological control agent efficiency.

A predatory mosquito larva of the genus *Toxorhynchites* is well-known biocontrol tool for *Aedes* mosquitoes because they share their larval habitats (Focks et al. 2014). Many species such as *Toxorhynchites splendens*, *Tx. brevipalpis*, *Tx. moctezuma* and *Tx. rutilus* are the efficient predator of *Ae. aegypti* (Yasuno and Tonn 1970; Padgett and Focks 1980; Sherratt and Tikasingh 1989). The populations of *Ae. aegypti* and *Cx. quinquefasciatus* were successfully reduced by weekly release of *Tx. amboinensis* in New Orleans, Louisiana, USA (Focks et al. 1985), and the same species has been evaluated in Fiji (Toohey et al. 1985) and Caribbean islands (Rawlins et al. 1991). However, production of large quantity of *Toxorhynchites* mosquitoes is hindered by their low reproductive capacity.

Other than predatory mosquitoes, the cyclopoid copepod crustaceans, *Mesocyclops* and *Macrocyclops*, are important biocontrol agents for *Aedes* mosquitoes. These copepods usually feed on first instars mosquitoes; *Mesocyclops* have reported reducing *Aedes* populations in Queensland Australia (Suarez et al. 1992; Kay et al. 2002) and French Polynesia. There is a report that *Mesocyclops* are prevalent in many *Aedes* larval habitats in Vietnam (Nam et al. 2000). Dengue cases have been significantly reduced by the application of *Mesocyclops* against *Ae. aegypti* control in Vietnam (Vu et al. 2005); the impacts on larval populations are persisted for several years (Kay et al. 2010).

The micro-biocontrol agents provide new dimensions with multiple ways of uses for better penetration to target habitats due to their small size and lightweight. The entomopathogenic fungi are one of the microbial agents that have been explored for the control of *Anopheles*, *Culex* and *Aedes* mosquitoes. For example, *Lagenidium giganteum* has been field evaluated against *Cx. tarsalis*, the vector of Western equine encephalitis and West Nile virus, in California, USA (Kerwin and Washino 1987). *Lagenidium giganteum* was reported to effectively reduce the container mosquito population *Cx. quinquefasciatus* and *Ae. aegypti* (Guzman and Axtell 1987; Rueda et al. 1990). Another fungal species, *Beauveria bassiana*, has shown a negative

impact on survival rate, blood-feeding success and fecundity of *Ae. aegypti* (Darbro et al. 2012). Recently, *Beauveria bassiana* to develop a novel autodissemination formulation to attack multiple stages of *Ae. aegypti* mosquito that suggested a high potential for the mosquito controls (Snetselaar et al. 2014). However, large field studies are needed to establish the autodissemination efficacy of these fungal species. The fungal spores help in the recycling of fungi in the treated habitat which provides an advantage over the insecticidal treatment because, in contrast to insecticides, the repeated use of fungi is not required (Hallmon et al. 2000). *Metarhizium* genus, a soil fungus, is lethal for mosquitoes. Two *Metarhizium* fungi, *M. anisopliae* and *M. brunneum*, have been evaluated for their pathogenicity. *Metarhizium anisopliae* infects both *Aedes* and *Culex* mosquito larvae through ingestion of conidia and later concentrate into the gut (Agudelo-Silva and Wassink 1984; Riba et al. 1986; Scholte et al. 2004), while *M. brunneum* infect mosquito larvae by adhering blastopores to the cuticle making *M. brunneum* more virulent than other species (Alkhaibari et al. 2016). The ability of rapid killing of mosquito larvae after penetration of blastospore or conidia makes these fungi a potential biocontrol agent.

The biocontrol agents *Toxorhynchites* mosquitoes, cyclopoid copepods, mermithid nematodes and fungi are effective tools of mosquito control. Nonetheless, mosquito larvivorous fishes may not be a feasible and preferred approach to control mosquitos found small containers.

11.5.4.5 Insecticidal Control of Larvae

The advantage of using larvicide and pupicide is substantial because of higher toxicity, easy applications in heterogeneous habitats and stability in the water. There is a large range of insecticides from non-specific to highly mosquito-specific. The fast-acting insecticides produce a result within 1–2 days, whereas slow-acting insecticides may take weeks to produce results. The fast-acting insecticides include organophosphates, pyrethrum and bacterium derivatives that are either neurotoxic or disruptive to the digestive system, whereas the slow-acting insecticides interfere with endocrine system regulation such as insect growth regulators. WHO (2009) has recommended the organophosphates (temephos and pirimiphos-methyl), slightly hazardous or unlikely to pose an acute hazard at normal use (1 ppm dosage) of larvicide. Insect growth regulator (IGR) is another category of WHO-recommended larvicides diflubenzuron, novaluron, rs-methoprene and pyriproxyfen. The first two IGRs are chitin synthesis inhibitors, while the last two are juvenile hormone analogues (Mulla et al. 1986). These IGRs are highly toxic to larval and pupal stages or transient stages showing 0.012 ppb as LC_{50} for pyriproxyfen against *Ae. albopictus* (Suman et al. 2014). Diflubenzuron has produced incompatible mosquitoes to biting after surviving at sublethal concentrations; the similar effect was not produced by azadirachtin IGR (Suman et al. 2010a, b). Pyriproxyfen acts at late larval or early pupal stages during metamorphosis and produced mortality during the pupal stage, known as pupicide; therefore, percent pupal mortality was used to estimate the effect of pyriproxyfen on mosquitoes in

larval bioassays (Suman et al. 2014, 2017). Biopesticides such as *Bacillus thuringiensis israelensis* (*BTI*) and spinosad at 1–5mg/l and 0.1–0.5 ppm dosage, respectively, have been suggested for the larval control (WHO 2009). Among these insecticides, rs-methoprene, pyriproxyfen and *BTI* can be used in potable water at recommended dosage (WHO 2009). The applicability of potable water makes them more effective because *Ae. aegypti* and *Ae. albopictus* mosquitoes preferably breed in fresh, potable and with low-organic-content water in or at the periphery of human populations.

Several laboratory and field studies have been conducted to evaluate the efficacy of various controlling agents including pyriproxyfen, BTI and other components (Suman et al. 2014, 2017; Williams et al. 2014). Basically, two type of larval habitat treatments have been conducted against *Aedes* mosquitoes in the field operations: (1) point-source/key habitat/hot spot treatment and (2) area-wide treatment.

Key habitats or hot spots such as junkyards, tyre piles, water tanks, construction sites and discarded household containers are the major mosquito-populated areas that contribute significantly to *Ae. albopictus* mosquito population buildup (Unlu et al. 2011; Fonseca et al. 2013; Suman et al. 2017). Targeting these key habitats may provide a significant reduction in mosquito population of treatment areas (Unlu et al. 2013). Under the point-source/key habitat treatment, a larvicide is applied to the localized habitats in a small area or most productive containers through the backpack or low-volume sprayers or by hand. For example, Suman et al. (2014) have shown significant mortality indirect treatment of pyriproxyfen in backyard parcels using backpack and ULV sprayers, although the backpack sprayers have extended the effectiveness up to 6 weeks in comparison to 3 weeks of ULV sprayer applications. Another study conducted by Scott et al. (2013) have shown the slightly better efficacy of ULV sprayers by using large size containers that may be because of the presence of more droplets in large containers. Key habitat treatment strategy is more eco-friendly and economical as it reduces the exposure of nontargeted areas and also required low quantity insecticides for the treatment in comparison to area-wide treatment.

In area-wide treatment, *BTI* emulsifiable larvicide formulation has been evaluated against *Ae. albopictus* using cold aerosol foggers and misting machines for operational area-wide applications in urban and semiurban habitats of New Jersey state, USA (Williams et al. 2014). The cold aerosol and conventional ULV sprayers for *BTI* application were unable to match the required flow rates for the area-wide treatment (Williams et al. 2014). Other sprayers such as Ag-Mister LV-8 orchard sprayer with eight nozzles (Curtis Dyna-Fog, Westfield, IN, USA) and Buffalo Turbine CSM2 mist sprayer (Buffalo Turbine, Springville, NY, USA) were found to be more suitable to deliver the required droplet size and flow rate for the peridomestic application of *BTI* larvicide (Williams et al. 2014). Buffalo turbine generates its own wind at the speed of 177 km/h making it less dependent on environmental conditions in comparison to passive wind reliant LV-8 mister to carry the droplets to the target habitats (Williams et al. 2014). The *BTI* application at 324 gm/acre provided more than 90% larval mortality for 6 weeks consistently (Williams et al. 2014). The enhanced efficacy of *BTI* can be directly related to the potency of the

formulations; VectoBac WDG contains 3000 international toxic units (ITU) per mg of *BTI* strain AM 65-52 than the VectoBac 12AS (1200 ITU/mg). Therefore, the VectoBac WDG is more toxic at the same rate making it more suitable for different applications because it requires less chemical volume (Farajollahi et al. 2013; Williams et al. 2014). Although VectoBac WDG is six times more expensive than VectoBac 12AS (Williams et al. 2014) and required 50–400 g/acre as per the label rate (Valent BioSciences, Libertyville, IL, USA), *BTI* may be an important larvicide because it is eco-friendly, a bacterial product that does not affect the nontarget insects due to its high specificity to mosquitoes and its lack of insecticide resistance (Lee et al. 2008; Lam et al. 2010).

Other insecticides such as insect growth regulators including juvenile hormone analogue (JHA) and chitin synthesis inhibitors (CSI) have also been found suitable for area-wide applications. These insecticides are also safe for vertebrates and non-insect organism due to the high affinity with insect system (Mulla et al. 1986). Methoprene is available in two emulsifiable formulations, Altosid SR-5 and Altosid SR-20, whereas pyriproxyfen has also been developed in granule/briquette formulation to extend its efficacy. IGRs are slower in action that affects the particular stage of life cycle during mosquito development and are cost-effective than *BTI* because it is chemically synthesized and formulated for the commercial production. Both methoprene and pyriproxyfen have been used effectively in various field trials in New Jersey, USA (Fonseca et al. 2013; Suman et al. 2014). Belinato and Valle (2015) have shown that the application of diflubenzuron IGR can be effective against the temephos-resistant *Ae. aegypti* populations; however, continuous selection up to seven generations elevated the tolerance to diflubenzuron (Belinato and Valle 2015). Contrary to this, Schaefer and Mulligan III (1991) did not find any sign of resistance development in *Cx. quinquefasciatus* larvae when continuously exposed to pyriproxyfen for 17 generations (Schaefer and Mulligan III 1991). The median emergence inhibition for these IGRs is at sub-ppb level (pyriproxyfen = 0.012 ppb) that reduced the application rate and flow rate which facilitate the conventional low-volume (LV) or ultra-low-volume (ULV) sprayers to be used for area-wide applications. The applications of LV and ULV machines in area-wide treatments enable the mosquito control cost-effective and affordable and avoid extensive labour involvement.

We and others have found that the effective treatment of sentinel containers used for the sampling does not necessarily represent the adult population reduction in the field and may not be effective for human landing reduction (Fonseca et al. 2013; Suman et al. 2014; Williams et al. 2014). This overestimation of efficacy may be attributed to open nature of sentinel containers which receive sufficient dose of insecticides, and they contribute a small proportion of the breeding habitats present in the field. *Aedes albopictus* prefer cryptic containers for oviposition, and, therefore, the delivery of insecticide into these containers is highly difficult (Unlu et al. 2014; Chandel et al. 2016). Chandel et al. (2016) have shown that cryptic containers are nearly impossible to spray chemicals via backpack and truck-mounted low-volume sprayers under field conditions (Chandel et al. 2016). Therefore, most of the area-wide applications cannot provide the reduction in adult mosquitoes or long-term suppression as cryptic habitat populations backup of the mosquito production.

Therefore, the advantage of larvicide and pupicide is significant to mosquito control due to their specific mode of action, easy availability, cost-effective and quick applications; however, there is always a threat for the development of insecticide-resistant because of insecticide's repeated applications. Despite the availability of effective insecticides, trained manpower and sufficient budget, the control of *Ae. aegypti* and *Ae. albopictus* required novel tools to deliver the controlling agents to achieve eco-friendly and persistent suppression of mosquito populations.

11.5.5 Adult Mosquito Biology, Surveillance and Management

The adult stages of both *Ae. aegypti* and *Ae. albopictus* mosquitoes are crucial because of their roles in biting and then transmitting the deadly diseases to humans. *Aedes* aegypti and *Ae. albopictus* known as day-bitter mosquitoes are primarily anthropophilic mosquitoes that have synchronized their blood-feeding rhythms with human activities (Hawley 1988; Faraji et al. 2014). The adult mosquito females survive for the almost 1-month duration in which they feed multiple times. The female mosquitoes supports pathogens survival and multiplication thereby increasing the parasitic load and then becoming infective. When an infected mosquito bites a healthy person for blood feeding, the parasite is injected into host bloodstream. Both *Ae. aegypti* and *Ae. albopictus* are efficient vectors of several arboviruses such as dengue, chikungunya and Zika, survive and propagate near human residential premises and fly within 50–100 m distance (WHO 2009, 2014). *Aedes aegypti* is more endophilic, whereas the *Ae. albopictus* is exophilic and reduces outdoor activities. Due to their diurnal rhythm, anthropophilic nature and peridomestic inhabitation, several methods of vector control become ineffective on these mosquitoes. Although several control measures and surveillance methods are used to monitor their density and reduce mosquito biting.

11.5.5.1 Surveillance

Because of anthropophilic, day activity and oviposition behaviours of *Ae. aegypti* and *Ae. albopictus*, adults of most of the traditional traps and sampling methods become ineffective that are difficult to use for their surveillance. For years, light traps have been efficiently used for the surveillance of *Anopheles* and *Culex* mosquitoes because they are nocturnal and attracted to the light source (Silver 2007). However, *Aedes* are day-active mosquitoes, and light trap may not be efficient to attract them; hence data will be underestimated and might not be useful for surveillance estimation. For the surveillance of *Aedes* adults, different traps have been developed that utilize skin emanations, known as kairomones, which mimic the presence of a human to lure host-seeking females for the blood feeding, i.e. chemical lure, CO_2 gas. Similarly, trap design has also played important role in attracting

females that include side opening and top opening(Wang et al. 2014). These are diurnal mosquitoes that can detect the colours and also their patterns (Hoel et al. 2009). The cumulative use of these cues may be used to develop efficient traps.

For the surveillance of adult *Aedes* mosquitoes, BG-Sentinel traps (Biogents Inc., Germany) have been evaluated extensively (Farajollahi et al. 2009; Suman et al. 2014; Williams et al. 2014; Unlu et al. 2017). This trap was developed initially for the surveillance of *Ae. aegypti* and found to be highly efficient (Maciel-de-Freitas et al. 2006, 2008; Ball and Ritchie 2010). This trap utilizes the chemical lure detected from the human or other host body emanations to attract the host-seeking female mosquitoes. Unlike to light traps that can be used during night hours only, BG-Sentinel trap can be used for 24 h covering both day and night photophases. This trap generates a plume around the trap and can effectively send cues to mosquitoes distant several metres. In addition, BG-Sentinel trap was evaluated for *Ae. albopictus* which produced the considerable efficacy of *Ae. aegypti* and has now been established as a gold standard technique. Comparatively, BG-Sentinel trap has been found to be efficient and can collect more *Ae. albopictus* adults than the Centers for Disease Control and Prevention (CDC) carbon dioxide-baited traps and other similar adult traps (Meeraus et al. 2008). BG-Sentinel trap is reported to be more efficient than the human landing catch in different conditions (Farajollahi et al. 2009; Hoel et al. 2009; Obenauer et al. 2010; Crepeau et al. 2013). Ritchie et al. (2006) have shown higher efficacy to detect new infestations of *Ae. albopictus* in Australia. In the USA, BG-Sentinel trap density has been utilized to establish the critical density for the hotspot identification as well as estimation of the intervention efficacy (Farajollahi et al. 2012; Unlu and Farajollahi 2014). In Italy and Brazil, BG-Sentinel traps deployment for trapping and surveillance have shown the reduction in *Aedes* population (Degener et al. 2014; Englbrecht et al. 2015). The other trap, mosquito magnet trap (American Biophysics Corporation, North Kingston, RI), has been evaluated against saltmarsh mosquitoes, *Aedes taeniorhynchus* (Kline 2006), and found to be effective to reduce *Aedes sticticus* and *Aedes vexans* population by 30% (Jackson et al. 2012). Mosquito magnet trap equipped with human scents and octanol lure performed superiorly than BG-Sentinel traps which have provided an alternative to BG-Sentinel traps (Rochlin et al. 2016). These traps can be used to estimate the density of *Ae. aegypti* and *Ae. albopictus* efficiently. However, further research on the development of better design and strong lures to attract mosquitoes may provide an eco-friendly tool to reduce the mosquito population.

11.5.5.2 Control Strategies for Adult *Aedes*

Various insecticides are effective to kill or knock down the adult *Aedes* mosquitoes to reduce their longevity and reproductive fitness (WHO 2009). There is a comprehensive list of WHO (2009)-recommended insecticides including organophosphates and pyrethroids which are moderately hazardous (Class II) and slightly hazardous (Class III) and unlikely to pose an acute hazard in normal use (Class U) (WHO 2006). Insecticidal control, particularly, adulticide application, has been recommended when

the environmental and non-chemical methods are failed or the situation is out of control as it creates environmental hazard significantly (WHO 2006). As per the recommendation and insecticide label, most insecticides can be used for both purposes as cold aerosols and thermal fogs except cypermethrin and D-phenothrin that can only be used in cold aerosols.

11.5.5.3 Thermal Foggers and Ultra-low Volume Sprayers

For adulticide applications, residual spray, thermal foggers or ULV sprays are found to be highly effective and efficient to reduce the mosquito population quickly in urban or semiurban conditions.

11.5.5.4 Residual Spray

The residual spray is conducted by hand-held or power-operated equipment to spray various insecticide formulations on different surfaces preferred by mosquitoes for resting. This spray produces long-term toxicity which results in the killing of adult-visiting mosquitoes to the treated zones. Sometimes, power or motorized sprayers can be utilized if treatment area is large, for example, tyre piles, junkyards, etc. The advantage of residual spray is that the toxic effect of the insecticide persists for the longer duration, unlike ULV or other broadcast sprays that do not persist for a longer duration. However, the residual spray is time-consuming and tedious and required significant good number of manpower to conduct door-to-door application. Moreover, there is a considerable hindrance for mosquito control team to access abandoned or locked private properties.

For the urban areas, area-wide ULV spray is an effective method which is easy to operate, provides large coverage area and cost-effective operation and does not require many people to perform the treatments (Farajollahi et al. 2012). However, ultra-low-volume spray needs optimization to get the best outcomes from the treatment for (1) droplet size should be 5–25 μm to contaminate mosquitoes, (2) occurrence of temperature inversion to allow droplets in air before settling down the ground, (3) wind speed to carry the droplets to target sites and (4) spray vehicle speed to adjust the flow rate (Haile et al. 1982; Mount 1998; Bonds 2012). In most cases, night hours are preferred for ULV spray to avoid human exposure and environmental conditions, but this may also result in lower efficacy due to the resting of *Ae. aegypti* and *Ae. albopictus* mosquitoes inside the house or in peridomestic habitats and in vegetation (Focks et al. 1987; Perich et al. 1990; Reiter 2007). Apart from these conditions, a single night hour truck-mounted ULV application of Duet™ insecticide reduced 73% *Ae. albopictus* population in New Jersey, USA, and a dual application with 1 or 2 day interval produced 85% reduction in the population (Farajollahi et al. 2012; Faraji et al. 2016). Duet™ has a dual action; it agitates the mosquitoes while in rest thereby killing them (Suman et al. 2012). A significant reduction of *Ae. aegypti* population has been observed by aerial spraying of mala-

thion in Florida and Louisiana, USA (Britch et al. 2008; Focks et al. 1987). Although the effects of ULV spray is transient and can provide relax for almost a week period by killing only the standing adult population during the spray, it remains an important tool for the intervention of container mosquitoes in urban conditions.

11.5.5.5 Barrier Residual Treatments

Container-inhabiting *Ae. aegypti* and *Ae. albopictus* are weak flyer mosquitoes and rest in domestic or peridomestic structures such as houses, containers and bushes. The treatment of resting places (vegetation, unmovable large containers, the external wall of home, sheds or focal points, etc.) with a barrier or residual insecticide spray helps in reduction of biting pressure with the suppression of adult population that may be an alternative strategy. This spray can be done by regular hand-held instruments including backpack sprayers, spray tanks, etc. (Cilek 2008; Trout et al. 2007; Unlu et al. 2017). The most preferred insecticide belongs to synthetic pyrethroid as it produces quick knockdown and allows low recovery (Trout et al. 2007; Dutta et al. 2017). The studies have shown that 85% of adult *Ae. albopictus* population were reduced with the barrier treatment of two pyrethroids, the residual effect persisted up to 1 month in Kentucky, USA (Trout et al. 2007). Similar results were obtained by Cilek (2008) in Florida, USA, that showed the reduction of mosquito population in treatment sites with the application of pyrethroids on vegetation (Cilek 2008). Abramides et al. (2011) have shown a reduction of *Ae. albopictus* population in public parks by treatments on vegetation in Spain. Another study conducted in China by Li et al. (2010) has also shown 98% reduction of *Ae. albopictus* population after treatment of pyrethroid on vegetation surrounding human houses. Contrary to these findings, Fonseca et al. (2013) could not find a significant reduction in adult population in New Jersey, USA, with deltamethrin barrier treatment (Fonseca et al. 2013). Recently, Unlu et al. (2017) have shown more than 70% reduction of *Ae. albopictus* population with barrier treatment of lambda-cyhalothrin alone and in combination with pyriproxyfen, and the effect of the treatments was noted to persist for 2–4 weeks. This indicates that the barrier treatment is an efficient technique to reduce the mosquito population that brings down the biting rates of mosquitoes, and, therefore, it can be preferred by homeowners to obtain the long-term efficacy.

11.5.5.6 Lethal or Autocidal Ovitraps

The ovitraps are known to attract gravid females and then hold a large number of eggs that have been utilized worldwide for the surveillance (Perich et al. 2003; Ritchie et al. 2009; Unlu et al. 2017). Later, ovitraps were redesigned and modified to capture the gravid females looking for a site to deposit the eggs (Ritchie et al. 2003; Mackay et al. 2013; Barrera et al. 2014; Johnson et al. 2017). These lethal or autocidal traps are equipped with insecticides to kill adults, sticky strips to capture

the visiting mosquitoes or unidirectional cone application for mosquito entry (Perich et al. 2003; Rapley et al. 2009; Ritchie et al. 2009, 2014; Mackay et al. 2013; Eiras et al. 2014). Studies conducted by several laboratories have shown that the trapping of gravid females using lethal ovitraps reduced the mosquito population of container *Aedes* mosquitoes effectively (Perich et al. 2003; Williams et al. 2007; Rapley et al. 2009; Zeichner and Debboun 2011; Barrera et al. 2014; Ritchie et al. 2014). Barrera et al. (2017) have shown a reduction in chikungunya positivity in *Ae. aegypti* with autocidal gravid trap implementations suggesting the possible role in arboviral disease prevention (Barrera et al. 2014, 2017). Although the results on these traps are encouraging, large-scale field trials in different conditions are needed to establish the lethal or autocidal ovitraps as a control strategy either used alone or as a part of integrated vector management (IVM).

11.5.5.7 Attractive Toxic Sugar Baits (ATSB)

Sugar as an energy source for survival and mating success is a crucial component for both male and female mosquitoes (Villiard and Gaugler 2015). In nature, mosquitos acquire sugar from various sources such as flower nectar, ripe and rotting fruits and blood (Foster 1995; Muller et al. 2011). The exploitation of sugar-feeding behaviour for the control option has been established as attractive toxic sugar bait (ATSB) method (Muller et al. 2010a, 2011; Marshall et al. 2013). To develop a sugar toxic bait, several chemicals were evaluated in a combination of toxicants such as boric acid, eugenol, garlic oil, dinotefuran, pyriproxyfen, spinosad and ivermectin (Muller et al. 2008; Xue et al. 2011; Khallaayoune et al. 2013; Marshall et al. 2013; Naranjo et al. 2013; Fulcher et al. 2014; Scott et al. 2017; Yaren et al. 2017). These ATBS were spread on vegetation where mosquitoes landed and possibly contacted to the treated surface thereby got exposed to the toxicant that leads to their eventual death. Several studies were conducted on *Anopheles* and *Culex* mosquitoes in various locations that showed significant reduction of mosquito vector population (Muller and Schlein 2006, 2008; Muller et al. 2010b; Qualls et al. 2012, 2014; Naranjo et al. 2013; Fulcher et al. 2014). A study using boric acid-based ATBS showed a significant decrease in *Ae. albopictus* population for 3 weeks and reduced the ovitrap catch for 2 weeks (Naranjo et al. 2013). When ATBS was sprayed on foliage, it produced higher reduction (85%) in comparison to the baited station (24%) against *Ae. albopictus* (Revay et al. 2014). Revay et al. (2014) have recommended that ATBS should be used on non-flowering plants to avoid exposure of nontarget insects (0.6%) (Revay et al. 2014). Efforts were also made to utilize the ATBS with a floral-based attractant to enhance the catch of *Ae. aegypti* (Fikrig et al. 2017); however, the efficacy is enhanced when L-lactic and 1-octen-3-ol were used in ATBS suggesting that it may be useful to develop new trapping device (Scott-Fiorenzano et al. 2017). These studies indicate that ATBS is a promising control agent to suppress the mosquito population by vegetation treatment. It may be used as an alternative to barrier treatment to avoid mosquito immigration from surrounding areas.

11.6 Novel Technologies for Multi-life Stage of *Aedes* Vector Control

11.6.1 Autodissemination Technology

Autodissemination is a novel mosquito management tool to deliver the control agents by conspecific individuals to their habitats that result in mortality or reduction of fecundity and fertility of the target insects. This technology is based on pull-push (attract-repel) strategy of insect controls. Autodissemination provides precise treatment of target sites making it more eco-friendly over broadcast spray of insecticides (Gaugler et al. 2012). Initially, autodissemination was tested against various insects utilizing different control agents including entomopathogenic fungi, baculoviruses and even nematodes (Baxter et al. 2009).

Recently, pyriproxyfen, a juvenile hormone analogue (JHA), is found to be highly lethal for *Aedes* mosquitoes at 0.012 ppb as LC_{50} against *Ae. albopictus* and *Ae. aegypti* (Sihuincha et al. 2005; Suman et al. 2014). The higher toxicity ratio provides an opportunity to develop a formulation with sufficient dose to contaminate few litres of water container effectively and can be carried by an adult mosquito for their flight range (Wang et al. 2014; Suman et al. 2017). Initially, Itoh et al. (1994) have shown the successful transfer of pyriproxyfen by gravid females (Itoh et al. 1994). Then, Dell Chism and Apperson (2003) have demonstrated that the sufficient pyriproxyfen could be loaded on the females by forcing the females to walk on pyriproxyfen-treated paper and subsequently transferred to ovicups that resulted in adult emergence inhibition up to 70% in *Ochlerotatus (Aedes) triseriatus* and 59–73% in *Ae. albopictus* in small-cage experiments (Dell Chism and Apperson 2003). Later, Devine et al. (2009) demonstrated Itoh's (1994) concept successfully in a field study and showed that *Ae. aegypti* females could produce 42–98% larval mortality in sentinel cups by disseminating the lethal concentration of pyriproxyfen from dusted cloth encircling the station (Devine et al. 2009). For the contamination of gravid females, Devine et al. (2009) used cloths dusted with ground granule formulation of pyriproxyfen (Devine et al. 2009). Snetselaar et al. (2014) have successfully used a combination of pyriproxyfen and *Beauveria bassiana* fungi against *Ae. aegypti* in autodissemination station.

The autodissemination efficacy is directly proportional to the density of gravid females, the carrier of pyriproxyfen (Devine et al. 2009). Gaugler et al. (2012) have developed a device, called autodissemination station. The efficacy of the device mainly depends on (1) design that facilitates mosquito entry and forces them to contaminate before exit; (2) formulation that easily contaminates mosquitoes, persists on the body and releases during oviposition; (3) oviposition attractant to lure gravid females to visit the station; and (4) self-sustainable for seasonal use without critical maintenance.

Based on above-mentioned criteria, a three-side arm opening autodissemination station was designed by Gaugler et al. (2012) that was loaded with pyriproxyfen powder formulation mixed with 10% emulsifiable concentrate (NyGuard® IGR,

MGK Chemical Co., MN, USA) as toxicant and oak infusion as an oviposition attractant (Trexler et al. 1998). The single station effectively attracted *Ae. albopictus* gravid females against two competing oviposition sites in the laboratory test and produced 100% emergence inhibition against four competing sites; however, the efficacy was slightly declined to 81%, in large area 31 m^3 (Gaugler et al. 2012). Interestingly, Gaugler et al. (2012) found that powder formulation was easy to clean off by mosquitoes and pyriproxyfen reduced to 0.324 μg/female from 0.524 μg/female (0 h) after 1 h of exposure and further declined to 0.044 μg, 0.016 μg and 0.018 μg at 12 h, 24 h and 48 h, respectively (Gaugler et al. 2012). These findings set a path to develop the new formulation that has longer persistence as mosquito visits several sites before reaching to oviposition in the field conditions.

With the strategy to enhance contamination of the females and device attraction, Wang et al. (2014) developed a novel technique of dual contamination in which oil and powder served as biphasic formulation (Wang et al. 2014). The stations were provisioned with a unidirectional path so that the mosquitoes are coated with oil first, and then allow them to walk over powder formulation due to the precise gap over the powder formulation band that restricts mosquito to fly out without touching the powder. This dual treated single station provided 100% pupal mortality against ten competing oviposition sites in a 31 m^3 room. A semi-field evaluation in the greenhouse with two stations showed 57.1% pupal mortality with 91.7% sites contaminations indicating the advantage of dual formulation strategy to enhance autodissemination efficacy (Wang et al. 2014). The field trials of dual treatment stations showed an effective dissemination of pyriproxyfen in sentinel cups (Suman et al. 2017; Unlu et al. 2017). Suman et al. (2017) studied the efficacy of autodissemination station to determine the impact of competition for oviposition sites, the density of stations, junkyard, tyre piles and peridomestic conditions which showed sufficient transfer of pyriproxyfen into sentinel cups (Suman et al. 2017). Suman et al. (2017) also suggested that pyriproxyfen can be transferred up to 200 m distance from the source station indicating a great potential to disseminate the control agent under field conditions (Suman et al. 2017). The pyriproxyfen residue liquid chromatography coupled with dual mass spectrophotometry (LC-MS-MS) analysis from sentinel cups detected up to 0.741 μg/l concentration which is more than enough to get significant mortality (Suman et al. 2017). In another study, a considerable mortality in sentinel cups was achieved with a simple design station dusted with ground pyriproxyfen granules (Caputo et al. 2012). However, long-term seasonal field evaluations are needed.

Moreover, managing *Aedes* mosquito populations in cryptic habitats has been a great challenge for vector control agencies. A field evaluation showed that the autodissemination station can successfully contaminate the cryptic sentinel containers with pyriproxyfen and produced significant mortality for several weeks. Interestingly, the same containers were resistant to the backpack and truck-mounted LV sprayer and could not be treated effectively (Chandel et al. 2016).

Instead of depending on field female population, Mains et al. (2015) have extended the idea of Gaugler et al. (2012) for the venereal transfer of pyriproxyfen and used male mosquitoes as a carrier for pyriproxyfen to contaminate females and

sentinel cups. This method has been described as "Auto-Dissemination Augmented by Males" (ADAM). The field release of pyriproxyfen-treated males was able to contaminate the lethal concentration in oviposition sites without the presence or absence of females at the site (Mains et al. 2015). The efficacy of ADAM that is independent of the number of females to carry the insecticide is a significant advantage over autodissemination station method and hence could be used in the pre-mosquito season like early spring to contaminate the habitats for proactive management. On the other hand, this method requires a huge number of males to cover a flight range of this mosquito. A male has a significantly shorter lifespan; therefore, accumulating a large number of young males and supplementing weekly release will require a large facility and logistic support for rearing, sorting and transporting with the contaminated device.

The autodissemination strategy is a promising future tool to manage *Ae. aegypti* and *Ae. albopictus* as well as other container mosquitoes as it has potential to deliver sufficient toxicant precisely to the target site regardless of open or cryptic in nature and up to their flight range. The ADAM method provides another advantage being independent of mosquito density for proactive management of *Ae. aegypti* and *Ae. albopictus.*

11.6.2 Genetic Control

11.6.2.1 *Wolbachia*: Cytoplasmic Incompatibility

The induction of cytoplasmic incompatibility (CI) in mosquitoes for the biological control tools is a unique and reliable method. *Wolbachia* species has been identified as an efficient organism for CI. *Wolbachia* is an intracellular gram-negative bacteria that can be maternally inherited (Calvitti et al. 2012; Zug and Hammerstein 2012; McGraw and O'Neill 2013; Lambrechts et al. 2015). The first *Wolbachia pipientis* was isolated from *Cx. pipiens* in 1924 (Hertig and Wolbach 1924). The main cause of cytoplasmic incompatibility is the survival of the bacterium in the reproductive organs. An abnormal embryonic development produces inviable progenies after the mating of an infected male with uninfected females. As the male is a dead-end host of *Wolbachia*, so viable progeny production can happen when infected female mate with an infected or uninfected male. *Wolbachia*-infected male produces sperm incompatible to fertilize the eggs from uninfected females or infected with different *Wolbachia* type (Dobson 2004). The maternal transfer of bacteria is an efficient mode of infection and can be utilized for gene-drive technology. The microinjection technique is suggested to transfect other uninfected vector species at embryonic stages making it more feasible (Braig et al. 1994). Cytoplasmic incompatibility has been used efficiently to curtail filariasis by suppression of *Cx. pipiens* complex population (Laven 1967; Curtis and Adak 1974; Brelsfoard et al. 2008) and can be used against *Ae. aegypti*, the vector of many arboviruses, found to be rarely infected with *Wolbachia* (Coon et al. 2016). *Wolbachia*-infected lines of *Ae. aegypti*, *Ae.*

albopictus and *Ae. polynesiensis* have been established with microinjection technique (Xi et al. 2005a, b, 2006). To suppress the wild populations of *Ae. polynesiensis*, a major vector of lymphatic filariasis, *Wolbachia*-infected mosquitoes have been utilized efficiently in South Pacific Islands (Brelsfoard et al. 2008; Chambers et al. 2011; O'Connor et al. 2012). In dengue-endemic areas, *Ae. aegypti* populations have been successfully targeted with *Wolbachia*-infected mosquitoes. A wMel strain-infected *Ae. aegypti* achieved stable and high-frequency infection of *Wolbachia* that replaced the dengue-transmitting population with *Wolbachia*-infected refractory population and refractoriness to dengue virus persisting in the field population (Hoffmann et al. 2011; Frentiu et al. 2014; Ye et al. 2016).

In *Ae. albopictus* mosquitoes, a superinfection of two strains of *Wolbachia*, group A and B, is reported which complicate the strategy of CI sterilization. Particularly, male with single *Wolbachia* strain infection is ineffective against dually infected female *Ae. albopictus*, and females infected by one strain may be sterilized by dually infected males; thus, the release of triple infected individuals has been suggested as the best approach to reduce the superinfected *Ae. albopictus* population in the field (Xi et al. 2005a).

Evidently, several workers have demonstrated that *Wolbachia* is able to block the infection of several flaviviruses including chikungunya and yellow fever viruses in *Ae. aegypti* (Moreira et al. 2009; Van Den Hurk et al. 2012; Walker et al. 2011). Trans-infection of native *Wolbachia* of *Drosophila melanogaster* is found to be effective to enhance tolerance to West Nile Virus infection in *Cx. quinquefasciatus* (Glaser and Meola 2010). To induce the resistance against viral infection, *Wolbachia* introduction to particular mosquito life stage is required; introduction in adult stage may not effective to induce resistance.

Moreover, the infection of *Drosophila simulans Wolbachia* strains, i.e. *wMelPop*, to *Ae. aegypti*, has reduced lifespan and altered blood-feeding behaviour which can limit the transmission of arbovirus as mosquito has to survive enough to complete the extrinsic incubation period to become infective (Salazar et al. 2007; McElroy et al. 2008; McMeniman et al. 2009). The *Wolbachia*-based technology has tremendous potential to block the disease transmission of dengue and other arboviruses by causing sterilization, development of refractory strains and reducing vector potential in crucial vectors such as *Ae. aegypti* and *Ae. albopictus.*

11.6.3 Sterile Insect Technique (SIT)

The idea of genetic control began with a concept of a release of sterile insect techniques (SIT) almost half a century ago (Knipling 1955). The SIT involves the reduction of insect population by the production of infertile eggs with the mating of sterile male unable to fertilize the eggs successfully. For the production of sterile males, the radiation exposure was used to induce chromosomal abnormalities that resulted in defective sperms. Variable impacts have been observed in several field trials that were conducted to control mosquito populations globally. Initially,

Anopheles quadrimaculatus trial was failed in Florida, USA, and no reduction in adult population was recorded due to the poor competitiveness of sterile males even after the release of 430,000 males over 48 weeks at two locations (Dame et al. 1964). A field trial of sterile male release on an island effectively reduced *Cx. pipiens quinquefasciatus* populations (Patterson et al. 1970). However, the similar results could not be achieved in India against *Cx. pipiens fatigans*, presently known as *Cx. quinquefasciatus* after the release of thiotepa-chemosterilized 38 million males. The major drawback of sterile male release study was that the wild males also introduced through new migration from untreated areas. Cytoplasmic and chromosomal translocation method of sterilization provided a better alternative to irradiation or chemical treatment which has shown effective *Cx. quinquefasciatus* population suppression after the release of 23 million males over 14 weeks in India (Curtis et al. 1982). In Kenya, a trial on *Ae. aegypti* was conducted in which 57,000 genetically altered males with chromosomal translocation release for 10 weeks were not effective for a longer time because of partial sterility (McDonald et al. 1977). A significant sterility in *Ae. albopictus* population was induced by releasing of 900–1600 sterilized males/hectare in Italy (Bellini et al. 2013). A simulation modelling suggests that SIT would be effective and economical in combating arbovirus vector mosquitoes (Alphey et al. 2011; Oliva et al. 2012). Sterile insect technique with the modern genetic tool may be a more effective strategy for the control of disease transmission and population reduction; however, the production of large number sterile males, quality control and fitness of treated males were the limiting factors for it to be an effective method (Dame et al. 2009; Alphey 2014).

11.6.4 Release of Insects Carrying a Dominant Lethal Gene (RIDL)

RIDL method is based on a dominant lethal gene expression to induce mortality. This method was tested as a proof of concept in *Drosophila melanogaster* (Thomas et al. 2000). In this method, a female-specific transcriptional system consists of cytotoxic lethal genes, and a tetracycline-sensitive transcription factor was used (Gossen and Bujard 1992). A controlled dominant gene expression provides more flexibility in control options by selecting the time of death and developmental stage of the offspring (Alphey 2014). It has been implicated in the Mediterranean fruit fly, *Ceratitis capitata*, using tetracycline-controlled transactivator (tTA) (Ogaugwu et al. 2013). In mosquitoes, a transcriptional system is controlled by a combination of a tetracycline operator and the minimal promoter of *Drosophila* Hsp70. The lack of female-specific promoter resulted in lethality in both males and females of transgenic mosquito line OX513A. The overexpression of tTA produced high mortality when tetracycline is not supplemented to the mosquitoes (Phuc et al. 2007).

In the Cayman Islands, 80% suppression of *Ae. aegypti* populations were recorded (Harris et al. 2012). In Brazil, a 6-week continuous field release pro-

gramme of the transgenic OX513A line showed significant 95% reduction in adult *Ae. aegypti* population; however, the effect was delayed and took several months to show population reduction after the first release (Carvalho et al. 2014, 2015). In another study in Panama, similar results were reported where it took 109 days to show a significant reduction (Gorman et al. 2016). To produce flightless phenotypes, a female-specific *Aedes Actin-4 (AeACT-4)* promoter provided an ideal candidate for a RIDL system in mosquitoes (Munoz et al. 2004). Such females are unable to escape the water habitat after eclosion from the pupae and die eventually there. The same *Actin-4* promoter (*AealbAct-4*) has also been isolated and characterized in *Ae. albopictus* to produce flightless phenotypes (Labbe et al. 2012). Further research development and field evaluations on RIDL techniques against different vectors in various ecological conditions will be needed to establish its efficacy for the control applications and regulatory authorities.

11.7 Human Behaviour Alteration to Avoid Biting and Personal Protection

The active period of *Ae. aegypti* and *Ae. albopictus* is highly synchronized with human daily activities. This synchrony provides many opportunities for interactions between mosquito and humans, and their interaction is facilitated by humans as they provide containers and other habitats to survive and propagate in their surroundings. Mosquito finds the host in close range due to peridomestic inhabitation. To avoid mosquito biting, the following methods can be helpful:

11.7.1 Housing Structures

A mosquito-resistant home can lead to less human-mosquito interactions by reducing entry of mosquitoes from peridomestic habitats that might result in less biting frequency. A housing can be turned into mosquito-proof by applying mosquito nets on windows and doors. Lindsay et al. (2003) have shown that installing a ceiling or closing the eaves protected people from malaria in Gambia by reduction of *Anopheles gambiae* inside the house, where the ceiling was found to be more effective than closing eaves (Lindsay et al. 2003). By closing large entry of home was found to be effective in reduction of malaria vectors, *An. gambiae* and *Anopheles funestus* in Mozambique (Kampango et al. 2013) and for several *Culex* and *Mansonia* species (Ogoma et al. 2010) which may also be applicable to reduce *Ae. albopictus* entry into homes. However, *Anopheles* is nocturnal and has different biting behaviour than the *Aedes* mosquitoes. The elimination of *Ae. aegypti* larval habitats such as bath, kitchen work, animals, plants, etc. may be helpful to reduce the indoor population. Efforts should be made to reduce water storage by improving

water supply, and water containers should be covered by mosquito-proof nets. The clutter of discarded containers, tyres, toys, bird pots, etc. surrounding homes or housing have been identified as most prevalent larval habitats for both *Aedes* species. These habitats can be easily maintained and mosquito proofed by unskilled persons also. Some permanent fixtures, such as rain gutter hose, are positive for larval breeding and therefore are needed to be cleaned time to time or should be provisioned for no water collection inside the pipe.

11.7.2 Clothing

Using insecticide-treated clothes, i.e. curtains, may also help in reducing population by killing the mosquitoes when they are exposed to treated cloths during resting. Kroeger et al. (2006) have shown a significant effect of curtain and water container cover treatments on reduction of dengue mosquito vector in Mexico and Venezuela. Recently, Orsborne et al. (2016) have shown that permethrin-treated cloths can provide a significant reduction in landing and biting rate of *Ae. aegypti* mosquito. A full-body coverage with long sleeves and trousers reduced 24% landing and 91% biting rates in comparison to partial body coverage with short sleeves and shorts with 26% and 49% reduction in landing and biting (Orsborne et al. 2016). Similar results were reported by DeRaedt Banks et al. (2015) and suggest that insecticide-treated clothing may play a significant role in reducing dengue transmission. Pyrethroids are safer and knock down the mosquitoes quickly than the other insecticides, and the recovery from pyrethroid's exposure is low (Suman et al. 2010b; Unlu et al. 2017). A cone bioassay with 3 min exposure to treated area can be used to evaluate the knockdown and mortality to consider the effectiveness of the treatments (WHO 2006). This indicates that pyrethroid-treated cloths may be a suitable way for the persons working outdoor as personal protection measures to avoid mosquito biting.

11.7.3 Repellents

Mosquito repellent opens a new dimension to restrict the vector-borne disease transmission by keeping away mosquitoes looking for the host or blood feeding. They are one of the most preferred and effective tools for personal protection against day-biting mosquitoes specifically *Ae. aegypti* and *Ae. albopictus* (WHO 2009). Various repellents have been evaluated by several workers against several mosquito species (Cilek et al. 2004). *N*,*N*-Diethyl-3-methylbenzamide (DEET): This is a broad-spectrum repellent and used against several blood-feeding insects such as mosquitoes, biting flies, flea and ticks. It is considered as a gold standard repellent used as a control to evaluate the efficacy of new compounds (WHO 2006). It was discovered by US Department of Agriculture (USDA) and patented by US Army in 1946.

Different types of products of DEET are available in the market such as lotions, oil based and sprays. Against *Ae. albopictus*, 6-h protection was recorded at 12.5% concentration which increased to 8 h at 25% concentration (Schreck and McGovern 1989). A new 35% DEET polymer developed by 3M Corp., USA, has extended the efficacy equivalent to 75% DEET repellency and provided 95% protection for 12 h (Schreck and Kline 1989). The repellents such as IR3535 (3-[*N*-acetyl-*N*-butyl]-aminopropionic acid ethyl ester) or Icaridin (1-piperidinecarboxylic acid, 2-(2-hydroxyethyl)-1-methylpropylester) have provided effective protection against *Aedes* mosquitoes (Cilek et al. 2004; Naucke et al. 2007; Sathantriphop et al. 2014). Lupi et al. (2013) have reviewed 102 publications on repellents and conclude that DEET is the best repellent for personal protection (Lupi et al. 2013). Plant oils have also shown repellent activities against *Ae. aegypti*, *Ae. albopictus* and other mosquito vectors; however, the efficacy was variable (Tawatsin et al. 2006; Tisgratog et al. 2016).

11.8 Conclusions

The vector-borne arboviral diseases such as dengue, chikungunya and Zika cause great loss to humanity. Dengue is highly lethal that can kill a person in short duration, while Zika produced severe permanent disability in newborn babies that is known as microcephaly. Chikungunya is not considered as lethal as other viral diseases; it can cause severe pain, and arthralgia doesn't allow the patient to move. There is no cure known for any of these arboviral diseases; medication can only provide symptomatic relief, although efforts are underway to identify vaccines against these viruses as yet there is no success in sight. Thus, mosquito vector control is the only tool to restrict the spread of these diseases by involving several tools and techniques because biting frequency is directly proportional to vectorial capacity and spread of disease transmission. *Aedes aegypti* and *Ae. albopictus* are the vectors of these arboviral diseases. Both of these mosquitoes are highly anthropophilic and diurnal and survive in peridomestic habitats. These habits of *Aedes* mosquitoes failed many surveillance and control strategies used for *Anopheles* and *Culex* mosquitoes. Host chemical cues and behaviour-based traps were developed for adult and egg surveillance. Both *Ae. aegypti* and *Ae. albopictus* are skip ovipositors and prefer to inhabit containers for oviposition and larval development. Most of these containers are cryptic in nature and hard to find them by mosquito control people or to be treated by conventional sprayers. For the control of these *Aedes* mosquitoes, different strategies have been developed over the time to manage larvae and adults. Source reduction of larvae is an eco-friendly method but cumbersome and hard to motivate people for door-to-door cleaning. Biological controls are effective against mosquitoes; container inhabitation of *Aedes* spp. may reduce the efficacy of bio-controls. Insecticidal control of larvae and adults is conducted through hand-held, backpack and truck-mounted sprayers for the treatment of key habitats and area-wide control measures. They were highly efficient to reduce the mosquito

population. Although these methods cause environmental damage to some extent, they may be effective during outbreaks of disease. To make mosquito control more effective and eco-friendly, novel methods have been developed such as autodissemination of insecticides (pyriproxyfen and fungus), Attractive Toxic Sugar Bait (ATSB), lethal ovitraps and genetic controls. Autodissemination technology provided a targeted delivery of insecticide in the larval habitats and has shown effective penetration to cryptic habitats of *Aedes* mosquitoes. Pyriproxyfen-treated male mosquito release, ADAM method, extended the concept of autodissemination to relax density dependence factor for the efficacy. Lethal ovitraps are advanced version of traditional ovitraps showing promising results in surveillance and management of *Aedes* population. Genetic control methods have gone a long way from competitive mating of sterile male to produce non-viable eggs to genetically modified mosquitoes to inhibit the parasitic development as well as stop the population production with dominant lethal gene activation or *Wolbachia*-mediated technology. In the coming future, these technologies may replace the conventional way of mosquito control. For immediate protection at personal level can be achieved by applications of repellents (synthetic/botanical). DEET has been found gold standard and can give 8 h of protection. Modification of house and treated clothing have also been evaluated for the biting protection. The prevention from the biting of infected mosquitoes can be the first-line management of the disease as these mosquitoes survive in close association with human.

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Chapter 12
Long-Lasting Insecticide-Treated Textiles Preventing from Mosquito Bite and Mosquito-Borne Diseases

Michael K. Faulde

Abstract Among all the vector-borne diseases occurring worldwide, mosquito-borne diseases prevail by far resulting in approximately 700,000 deaths from clinical complications annually. Although malaria still accounts for the highest disease burden, currently emerging and resurging mosquito-borne viral diseases like dengue, West Nile, chikungunya, Rift Valley and even yellow fever viruses became either epidemic or pandemic, affecting many regions in the world. In February 2016, the World Health Organization declared the Zika virus public health emergency of international concern following large outbreaks with rapid geographical spread in the Pacific and Southern America, while Mayaro, Wesselsbron, Usutu and St. Louis encephalitis viruses have been identified as new disease agents showing pandemic potential.

The transmission of mosquito-borne disease agents can be readily interrupted by prevention from potentially infective mosquito bites. Therefore, personal protective measures against bites of hematophagous vectors constitute the first line of defence against mosquito-borne diseases. Besides the use of skin repellents and bite-proof textiles, long-lasting insecticide-impregnated bed nets and clothing have been developed during recent years which synergistically contribute to optimized personal protection. The aim of this study is to give an overview on the most current and widely used textile impregnation techniques, their efficacy in public health protection as well as their mosquito bionomic-specific use against daytime-, night-time-, indoor- and/or outdoor-biting vector mosquitoes. We strongly recommend the use of long-lasting permethrin-impregnated clothing for the prevention of mosquito-borne diseases transmitted by daytime- and night-time-active-, indoor- and outdoor-biting mosquitoes, including chikungunya, dengue and Zika fevers combined with the extensive use of long-lasting insecticide-treated bed nets preventing

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primarily from nocturnal, anthropophilic, indoor-biting mosquitoes vectoring, e.g. malaria, lymphatic filariosis and West Nile fever.

Keywords Permethrin-impregnated clothing · Insecticide-treated bed nets · Malaria prevention · Long-lasting activity · Personal protection · Mosquito bite protection · Public health implications

12.1 Introduction

Currently, 11 major global vector-borne human diseases have been identified by the World Health Organization (WHO) which results in approximately 17% of the estimated burden of communicable diseases, thus accounting for more than 700,000 deaths associated with these diseases (WHO 2017a). Among these diseases defined, approximately two thirds, namely, malaria, dengue, lymphatic filariosis, chikungunya, Zika virus disease, yellow fever and Japanese encephalitis, are mosquito-borne (GBD 2015; WHO 2017a). When compared, malaria still accounts for more than 60% of the estimated or reported number of annual cases of mosquito-borne diseases as well as for almost 96% of disease-related deaths which are estimated to sum up to 447,860 fatal cases per year (WHO 2017a). According to this analysis, malaria is still the most frequent mosquito-borne disease worldwide. Especially for the sub-Saharan Africa region, anthropophilic and endophagic anopheline vector mosquitoes currently are of biggest concern (WHO 2014).

Nevertheless, mosquito-borne viruses like West Nile, dengue, chikungunya and Zika viruses are emerging quickly and globally resulting in extreme outbreaks during recent years in known endemic as well as in formerly nonendemic areas followed by introduction either of the disease agent with transmission to indigenous or newly introduced vector mosquito species. The risk of human infection for these viral diseases is primarily associated with vector mosquito-specific behavioural aspects, bionomics and preferred environment. West Nile, dengue, chikungunya and Zika diseases are transmitted either by daytime-biting endophageous *Aedes* or nocturnal indoor-biting *Culex* mosquitoes, especially confined to, and prevailing, in an urban environment (WHO 2014). The majority of the globally emerging mosquito-borne viral diseases like dengue, chikungunya and Zika fevers are *Aedes*-borne, primarily and effectively transmitted by the yellow fever mosquito, *Aedes (Stegomyia) aegypti*, as well as the Asian tiger mosquito, *Ae. albopictus (Stegomyia albopicta)* (GBD 2015; WHO 2017b, c, d). Unlike sylvatic *Aedes* species, these two species are weak flyers and favour anthroponotic environments and urban areas.

Since 2014, major outbreaks of dengue, malaria, chikungunya and yellow fever have been reported in many countries worldwide afflicting billions of people while claiming lives and overwhelming the public health systems in many countries. Since the first detection of autochthonous transmission of chikungunya fever in Saint Martin in December 2013, more than 2.9 million suspected and confirmed cases including 296 deaths have been reported in the Americas until late July 2016 (Yactayo et al. 2016). Following the fulminant rise and spread of chikungunya virus

in the new world, Zika virus—primarily transmitted by the very same mosquito vector, *Aedes aegypti*—raged in South America, the Caribbean and beyond since its first occurrence in Brazil in late 2014 (WHO 2017b, d). Since 2015, hundreds of thousands of cases occur each year in the Americas, linked with clinical—especially neurological—complications like microcephaly and Guillain-Barré syndrome (WHO 2017d). Following its first introduction, the Zika virus' incredibly high average speed of spread within Brazil was estimated to be 42 km/day or 15,367 km/year (Zinszer et al. 2017).

While the WHO (2017c) calculated 3.2 million cases of disease of which 500,000 are severe leading to 12,500 deaths annually, Bhatt et al. (2013) estimated 390 million new dengue virus infections occurring annually worldwide. According to this data, dengue currently represents the by far most frequent mosquito-borne viral disease in the world thus increasing the public health impact of *Aedes* mosquitoes (Stanaway et al. 2016). While mosquito-borne viral diseases are currently quickly emerging globally, the number of annual malaria cases and associated deaths reported worldwide are steadily decreasing during recent years with 212 million annual cases and 429,000 deaths from malaria estimated for 2015 (WHO 2016). New studies reveal that rather rare and yet neglected mosquito-borne viruses show a high potential for emerging into the human population on a larger scale, among them Mayaro virus (Hotez and Murray 2017) as well as the Wesselsbron, Usutu and St. Louis encephalitis flaviviruses (Smith 2017).

When analysing the increasing number of vector-borne diseases of public health impact which are currently emerging or resurging worldwide, only few are effectively vaccine-preventable. Chemoprophylactic drugs are available only as a means of secondary prevention for malaria, but multidrug resistance is on the increase and spreading, especially in southeastern Asia and Africa. For this reason, personal protective measures against hematophagous vectors constitute an effective primary prevention method against arthropod-borne diseases in endemic areas (Faulde et al. 2006).

Since decades, the synergistic use of a safe and appropriate skin repellent formulation combined with long-lasting insecticide-treated fabrics, including clothing, tents and netting, is highly recommended for the protection of at-risk personnel exposed to vector arthropod-infested areas (WHO 2001a, b; Faulde and Uedelhoven 2006; Pennetier et al. 2010; Banks et al. 2014). Until recently, combined personal protective measures have been primarily employed individually during occupational-, travel- or leisure time-related exposure in the field. This philosophy changed markedly during the current Zika virus epidemics still occurring in the Americas when the extensive use of *N*,*N*-diethyl-meta-toluamide (DEET) skin repellent in combination with clothing, impregnated with the synthetic pyrethroid permethrin, has been strongly recommended as a public health core strategy to mitigate the spread of this disease within the affected human population (Wylie et al. 2016). Following detailed toxicological analyses, this method has been highly recommended by the United States Centers for Disease Control for Zika virus-exposed women, especially during pregnancy, in order to prevent from the devastating neuropathological consequences for foetuses (Wylie et al. 2016).

The aim of this study is to (a) give an overview on current knowledge, growing impact, principles of action and efficacy and (b) to analyse and discuss optimization options for use and development, shortcomings and future research fields of insecticide-treated textiles designed for mosquito bite protection in the public health sector.

12.2 Insecticide-Impregnated Clothing

The use of insecticides for fabric impregnation aiming at disease prevention is not new. Since Napoleonic times, it is well known that soldiers are highly vulnerable to infestations with body lice and, consequently, to epidemics of louse-borne diseases (Faulde 2006; Pagès et al. 2010). First attempts were made during World War II to treat combat uniforms with the newly developed organochlorine-insecticide dichlorodiphenyltrichloroethane (DDT) by using the dipping technique or simply by dusting individuals directly. Primarily, the aim was to control body louse infestations in order to prevent from potentially infected lice bites and mitigate further spread of louse-borne diseases (Faulde 2006). During the 1940s, it has been quickly realized that insecticide-treated clothing also prevent from mosquito, flea and other arthropod vector bites because military populations are frequently deployed to active vector-borne disease foci while experiencing an increased risk of contracting vector-borne diseases (Pagès et al. 2010). During the 1970s, DDT has been banned by many, especially industrial nations which made DDT impregnation of clothing obsolete. Fortunately, the first residual synthetic pyrethroids like permethrin have been developed at that time which had the potential to replace DDT for clothing impregnation purposes until today (Faulde et al. 2003). Because of its highly advantageous properties which include excito-repellency, hot-feet, knockdown, kill, high residual activity, laundering resistance and user safety, the synthetic residual pyrethroid permethrin has been widely used until today as an effective arthropod contact repellent for fabric impregnation (US Armed Forces Pest Management Board 2009; Vaughn et al. 2014; Faulde et al. 2016). Currently, permethrin is the only recommended and used synthetic insecticide for the impregnation of clothing.

To date, different methods for fabric impregnation with permethrin have been developed, all specifically affecting essential parameters like protective efficacy, residual activity, laundering resistance and bioavailability as well as homogeneity of distribution of permethrin molecules. Known and widely used methods are:

- The absorption method, wherein fabrics are individually treated by dipping or spraying ready-to-use permethrin solutions (Evans et al. 1990; Carnevale and Mouchet 1997).
- The incorporation method, known also as “Eulanisierung”, which uses heat and salt gradients to bind permethrin into wool or silk fibres and, most frequently, carpets (Zimmermann and Höcker 1988).

- The polymer-coating or foularding method, achieved by embedding the permethrin into single or multiple plastic layers by polymerization onto the fabrics prior to the tailoring process (Faulde et al. 2003).
- The treatment of fabrics by dipping or spraying methods with various types of micro- or nanocapsules containing permethrin (Yao et al. 2015; Forgearini et al. 2016).
- The permethrin micro-/nanocapsule-enhanced polymer-coating (or foularding) method combining the specific advantages of micro-/nanocapsule and polymer-coating impregnation systems allowing to extremely increase the long-lasting efficacy (Faulde unpublished).

The last three methods have been developed only recently in order to (a) avoid toxicological and logistical shortcomings of self-impregnation while saving the environment from contamination of residues stemming from the manual impregnation process; (b) increase user safety by reducing the migration rate of permethrin, which is embedded in plastic layers and/or micro-/nanocapsules, into the human body; (c) increase long-lasting efficacy by enhancing residual bioactivity and laundering resistance; and (d) avoid inhomogeneity of permethrin surface concentrations which could reduce the protective efficacy. It has to be considered that each impregnation method as well as any changes regarding treatment parameters may directly and substantially affect the delicate balance between the insecticide's bioactivity, bioavailability, migration rate, surface homogeneity, laundering resistance and residual activity as well as dermal exposure rates, transdermal migration, physiological incorporation and, therefore, user safety and bite protection efficacy (Faulde et al. 2003, 2016).

To date, most studies on impregnated fabrics efficacy have been conducted under laboratory conditions prior to and after laundering or under experimental conditions. The protective effect of impregnated clothes against mosquito bites has, however, been demonstrated in field or near-field conditions in two recent studies (Londono-Renteria et al. 2015; Osborne et al. 2016). Two additional studies have attempted to investigate the protective effect of permethrin-impregnated clothing against mosquito-borne diseases under field conditions in disease-endemic areas (Soto et al. 1995; Most et al. 2017). In a randomized, double-blind study published by Soto et al. (1995), Colombian soldiers wore permethrin-impregnated battle dress uniforms under noncombat conditions during a 4–6 weeks' period resulting in an incidence reduction rate of 80% against malaria and 78% against cutaneous leishmaniasis. Combat uniforms have been impregnated by a spraying technique which was commercially available on the US American market. Unfortunately, permethrin concentrations, residual activity, bioactivity, laundering and environmental effects were not monitored during this relatively short field study. A more recent investigation performed by Most et al. (2017) analysed the preventive effect of factory-treated long-lasting polymer-coated permethrin-impregnated clothing against malaria infection after military worst-case exposure to high-level disease transmission sites in the rain forest of French Guiana. Between August 2011 and June 2012,

25 personnel wearing impregnated clothing and exposed for 9.5 person-months in hyperendemic foci contracted no cases of malaria, whereas 125 persons wearing untreated uniforms only, exposed for 30.5 person-months, contracted 11 cases of malaria. The use of impregnated clothing significantly protected against malaria by reducing the malaria incidence rate more than 3.4-fold. Impregnated uniforms were laundered up to 218 times. 4.3% of blouses and 13% of trousers showed insufficient protective efficacy when tested according to the standardized licencing algorithm TL 8305-0331 due to permethrin loss associated with frequent laundering, mechanical abrasive processes and weathering. Although the polymer-coating technique allows high-residual long-lasting binding of permethrin onto the fabric, experiences made after long-term worst-case use in hyperendemic disease foci in rain forests revealed the need for an optimized impregnation method allowing an even higher residual activity as well as increased stability against laundering, abrasion and weathering processes (Most et al. 2017). Preliminary data of another recent study among migrant rubber tappers wearing permethrin-impregnated clothing in malarious areas in Myanmar showed a high acceptability among users as well as reduced mosquito biting rates (Crawshaw et al. 2017). No information is yet available concerning the preventive effect against malaria infections under the given study conditions and locations. Furthermore, no data is yet available on the protective efficacy of long-lasting permethrin-impregnated clothing against *Ae. aegypti*- and *Ae. albopictus*-borne viral diseases like dengue, chikungunya and Zika fevers during exposure in hyperendemic areas or during epidemics in the field.

12.3 Mode of Action of Insecticides and Repellents Used for Textile Impregnation

Until today, five chemical classes of synthetic insecticides are used to control adult mosquito vectors within the public health sector: organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids. All of them are acting as acute neurotoxins by either targeting the acetylcholinesterase (organophosphates, carbamates), the nicotinic acetylcholine receptor (neonicotinoids) or the voltage-gated sodium channels (pyrethroids, organochlorides) within the neuronal system. Insecticides usually affect—to a higher or lesser extent—the neurologic system of both target animals like insects and other arthropods and vertebrate nontarget animals including man. The higher the toxic effect of an insecticide to target animals and the lower its toxic impact to humans and other nontarget organisms, the higher its selectivity and, consequently, safety for the user and the environment. Generally, organochlorides, organophosphates and carbamates show a lower selectivity rate whereas pyrethroids and neonicotinoids are characterized by a higher selectivity (=safety) rate. These characteristics principally make pyrethroids and neonicotinoids excellent candidates for the safe use of impregnated textiles worn close to or directly on the human body. Unlike the “old” pyrethroids which have been developed in the 1970s, the newest class of insecticides, the neonicotinoids, are currently

discussed controversially due to their accumulating toxic effects in the environment. It has been proven that neonicotinoid use is strongly linked to the currently widely experienced honeybee population devastation while simultaneously affecting many other pollinators (Lundin et al. 2015). It is, therefore, unclear whether licencing of neonicotinoids will be extended in the future and whether they will remain commercially available for textile impregnation purposes on the European and/or American market.

Consequently, ester-, non-ester and α-cyano pyrethroids are used most widely for the impregnation of textiles, especially long-lasting insecticide-impregnated bed nets (Faulde et al. 2012). Among them, permethrin, etofenprox, deltamethrin, cyfluthrin, λ-cyhalothrin and α-cypermethrin are recommended by the WHO for the treatment of mosquito nets at substance-specific application concentrations (WHO 2013), and to date, permethrin remains the only recommended pyrethroid for clothing impregnation including mattresses (US Armed Forces Pest Management Board 2009; Vaughn et al. 2014; Faulde et al. 2016).

Usually, arthropods try to actively avoid direct exposure to contact pyrethroids by showing a so-called "hot-feet" effect including an enhanced motility reaction (Hoffmann 1995). The resulting excito-repellency effect is finally leading to reduced landing, probing and biting behaviour by vector mosquitoes on pyrethroid-treated clothing when using the arm-in-cage test (Faulde et al. 2012; DeRaedt Banks et al. 2015). However, when blood feeding is easily possible under laboratory conditions, e.g. bites through pyrethroid-impregnated bed net fabric characterized by a smaller or larger mesh size which has been wrapped around the forearm in order to expose the skin, mosquitoes readily feed on commercially available LLITNs despite direct contact to and intoxication with insecticides as well as excito-repellency effects (Faulde et al. 2012). For example, Fig. 12.1a shows *Ae. aegypti* mosquitoes biting through newly purchased, unlaundered PermaNet 2.0® bed net fabric treated with 55 mg permethrin/m^2, whereas Fig. 12.1b depicts this effect against the Conmanet® impregnated with 25 mg deltamethrin/m^2, and Fig. 12.1c shows an own polymer-coated research net containing 2000 mg etofenprox/m^2, all tested against the negative control (Fig. 12.1d) using the arm-in-cage test. Although excitatory behaviour of the test mosquitoes was documented on insecticide-treated netting, the time of exposure to the insecticide during the complete feeding process, which takes between 150 and 329 s (mean, 240 s) (Gillett 1967), was long enough to allow >95% mortality of all fed mosquitoes within the following 24 h when using the etofenprox-treated fabric. Although containing tenfold of the WHO-recommended etofenprox concentration of 200 mg/m^2 landing, probing and biting count of test mosquitoes did not differ from that of the negative control (Fig. 12.1c, d) (Faulde et al. 2012). Obviously, direct access to the skin as well as successful probing and blood feeding overcame the excito-repellency effect of insecticides when topically exposed in a test cage which finally lead to an increased exposure to contact biocides together with increased mortality in test mosquitoes.

New innovative insecticide combinations and treatment methods are necessary in order to overcome the growing lack of protective effect of long-lasting insecticide-treated bed nets (LLITNs) in geographic areas where mosquito resistance mechanisms are prevalent. Insecticide class-specific resistance mechanisms against

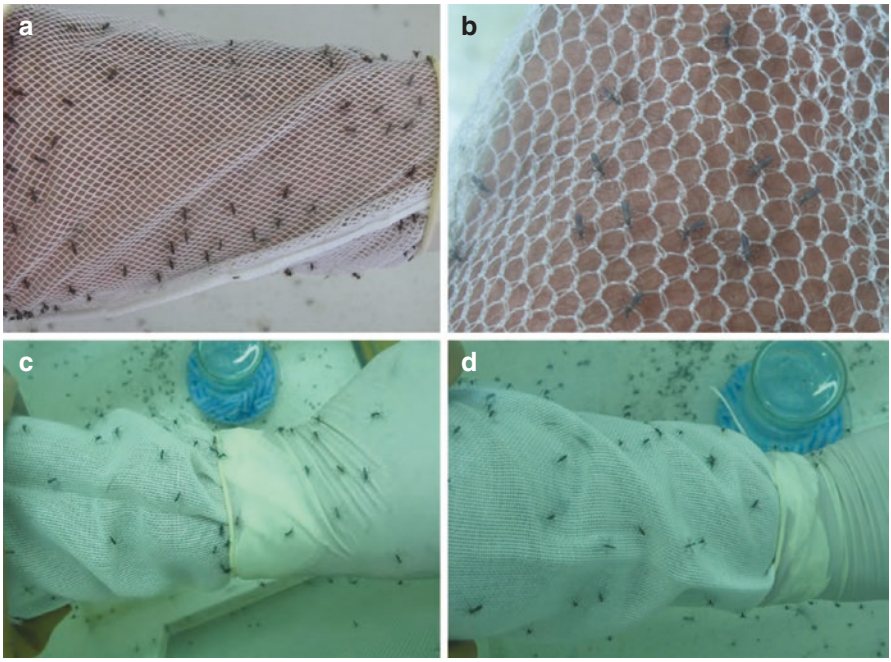

Fig. 12.1 Arm-in-cage tests showing landing, probing and biting/feeding behaviour of *Aedes aegypti* mosquitoes exposed to two brands of commercially available LLITNs and one type of research textile vs. negative control after 2 min of exposure: (**a**) PermaNet 2.0®; (**b**) Conmanet®; (**c**) research fabric treated with 2000 mg etofenprox/m^2; (**d**) untreated fabric (negative control)

pyrethroids include knockdown resistance mechanisms, caused by *kdr* and *ace-1R* gene mutations, as well as increased production of detoxification enzymes (Marius et al. 2017). In order to neutralize resistance mechanisms, three different main approaches are currently favoured which are (a) fabric impregnation with insecticide classes, e.g. carbamates, organochlorides or neonicotinoids, which overcome pyrethroid-specific resistances (Guillet et al. 2001), (b) the simultaneous use of the pyrethroid synergist piperonyl butoxide (PBO) (Marius et al. 2017), and (c) the combined use of pyrethroid contact insecticides together with skin repellents, like *N*,*N*-diethyl-m-toluamide (DEET) or insect repellent 3535 (IR3535) (Faulde and Nehring 2012).

Like residual pyrethroids, carbamates act as contact insecticides due to their low volatility. Furthermore, carbamates are known to be extremely effective in areas where highly pyrethroid-(*kdr*-) resistant vector mosquitoes are endemic (Djénontin et al. 2009). Carbamates used on bed nets or tarpaulins were carbosulfan at a concentration of 300 mg/m^2 as well as bendiocarb at 100 and 200 mg/m^2 (Guillet et al. 2001; Djénontin et al. 2009). Although highly effective against pyrethroid-resistant mosquitoes, carbamates show—when compared to pyrethroids—on average a markedly higher acute human toxicity and a considerably lower selectivity rate. Both parameters directly affect the user safety. Consequently, about 20% of sleepers

in that study reported potential toxicological side effects, like headache and sneezing, when using a carbosulfan-treated bed net (Guillet et al. 2001). Due to the toxicological impact for human health combined with a widely occurring *ace-1R* mutation associated with carbamate and organophosphate resistance, long-lasting carbamate-impregnated bed nets are currently neither available commercially nor recommended for use.

Another new attempt is to impregnate fabrics with a PBO-pyrethroid combination in order to increase the effectiveness of LLITNs in areas characterized by simultaneously occurring *kdr*- and *ace-1R* mosquito resistance mechanisms as well as detoxification enzyme upscaling. PBO is a well-known synergist which is widely used in combination with pyrethroids for insect pest control. The primary mode of action of the methylenedioxyphenyl compound, PBO, is to inhibit P-450 monooxygenase enzymes, also known as the mixed-function oxidase system (MFO). The MFO system is the main route of detoxification in insects and causes the oxidative breakdown of insecticides such as natural pyrethrins as well as chemically altered synthetic pyrethroids. Consequently, when PBO is added to synthetic pyrethroids, higher insecticide levels remain in the insect body which enhance their lethal effect (Moores et al. 2009). Different brands of new generation LLITNs, coated with pyrethroids and PBO, are now available on the market. According to a recent study, Olyset Plus®, coated with 2% (w/w) permethrin and 1% (w/w) PBO, and PermaNet 3.0® treated with 2.8 g/kg ± 25% deltamethrin plus 4 g/kg ± 25% PBO both showed better protective efficacy when compared to conventional LLITNs in malarious areas with a high vector mosquito resistance level in Benin (Marius et al. 2017).

In order to increase the protective effect of LLITNs in geographic areas where mosquito resistance mechanisms are widely occurring, the additional use of effective skin repellents is considered essential and is strongly recommended in order to ameliorate bite protection (WHO 2001a; Norris and Coats 2017). Primarily, licenced synthetic and safe skin repellent compounds like DEET, IR3535, KBR 3023/Picaridin/Icaridin/Saltidin or para-menthane-3,8-diol (PMD) are widely in use, known for their high repellent activity against mosquitoes, user friendly and are considered toxicologically safe (WHO 2001a, b; Faulde 2010). Many efforts have been undertaken in the past to treat clothing, bed nets and tents with skin repellents, especially with DEET, by using the spraying or dipping technique (Faulde 2010). Because molecules of skin repellents are usually characterized by a high natural vapour pressure, the active compound evaporates freely into the atmosphere where the chemicals can be smelled and avoided by mosquitoes. Therefore, skin repellents are characterized by a spatial activity (Faulde 2010; Norris and Coats 2017). Consequently, even when used at higher concentrations, the average bite protection time does not exceed 6–8 h when applied onto the skin or fabric due to complete evaporation of the molecules into the atmosphere (Fei and Xin 2007; Faulde et al. 2010). Durability of the repellent effect could be enhanced by employing microencapsulated DEET on cotton fabric. During graft copolymerization of butyl acrylate onto chitosan in an aqueous solution, resulting DEET microcapsules revealed 100% repellency for 8 h, including a partially preserved repellent activity for up to 48 h (Fei and Xin 2007). Another method to produce LLRTNs has been developed by

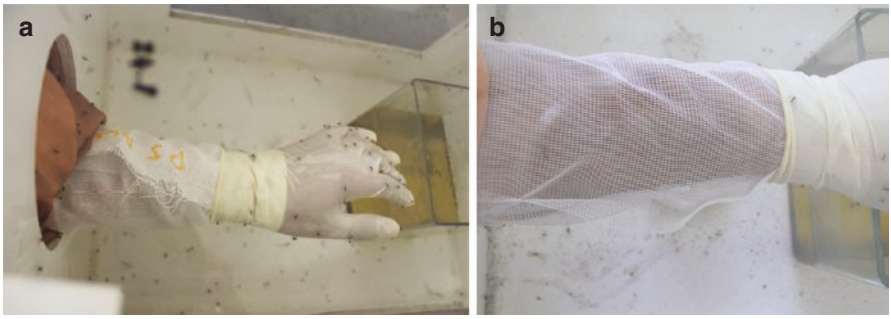

Fig. 12.2 Arm-in-cage tests showing long-lasting complete (100%) landing, probing and biting/feeding protection against *Aedes aegypti* mosquitoes after 5 min of exposure using skin repellents DEET and IR3535 together with permethrin and etofenprox, all polymer-coated onto textiles: (**a**) textile containing 3590 mg/m^2 DEET plus 1208 mg/m^2 permethrin; (**b**) bed net fabric containing 5930 mg/m^2 IR3535 plus 2139 mg/m^2 etofenprox. Notice landing and probing behaviour on the bite-protecting glove, serving as host-seeking control

binding DEET, or IR3535, onto fibres of bed net fabric using the polymer-coating technique (Faulde et al. 2010). By polymerizing multilayers onto the fibres in which the repellent molecules have been embedded within the plastic coating, extremely high DEET and IR3535 concentrations >10 g/m^2 could be obtained. One hundred per cent repellency, measured by complete landing, probing and biting protection using the arm-in-case test, could be achieved at DEET concentrations >3.7 g/m^2 (Fig. 12.2a) as well as for IR3535 contents >10 g/m^2 (Abb. 2b) (Faulde et al. 2010). One hundred per cent landing, probing and biting protection could be achieved with DEET-impregnated fabrics for 29 weeks at an initial concentration of 4.66 g/m^2, 54 weeks at 8.8 g/m^2, 58 weeks at 9.96 g/m^2 and 61 weeks at 10.48 g/m^2 as well as for 23 weeks using IR3535-coated fabric at a concentration of 10.02 g/m^2 (Faulde et al. 2010). In spite of the highly promising long-term protective efficacy of DEET and IR3535 polymer-coated onto bed net fabrics, laundering stability was, unfortunately, extremely low, resulting in a loss of the 100% repellency in treated clothing after the first washing process according to EN ISO 6330:2000 (International Organization for Standardization 2012), thus making its use for cloths obsolete in case frequent launderings are necessary (Faulde unpublished).

Besides behavioural aspects including spatial repellency, insecticidal effects of the skin repellents DEET, IR3535 and KBR 3023 have been detected when tested against *Ae. aegypti* (Licciardi et al. 2006; Pridgeon et al. 2009; Faulde et al. 2010). Obviously, these skin repellents do not behave like a homogeneous class of compounds expressing a single mode of toxic action. DEET, for example, exhibits more complex insecticidal properties when compared with the other chemicals. Although their detailed molecular and physiological mechanisms of action remained unknown, toxicological studies revealed that the mode of action of skin repellents differs markedly from that of pyrethroids and other insecticide classes (Licciardi et al. 2006; Faulde et al. 2010). Figure 12.3a shows the knockdown and kill effects of fabric impregnated with 0.5 g DEET/m^2 after 120 min of exposure against *Ae.*

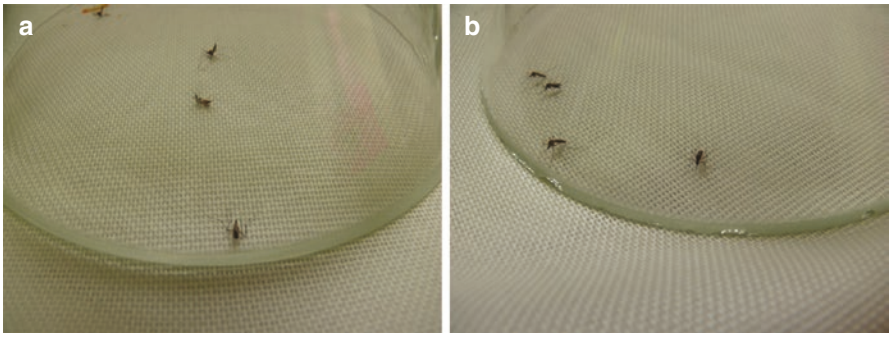

Fig. 12.3 Cone test documenting insecticidal effects of the skin repellent DEET at a—relatively low—concentration of 0.5 mg/m^2 against *Aedes aegypti*: (**a**) knockdown and kill effects after 120 min of exposure; (**b**) continuous contact of test mosquitoes to the treated surface after 5 min of exposure

aegypti mosquitoes following continuous contact exposure (Fig. 12.3b) using the cone test. Synergistic insecticidal and repellent effects have been documented when testing combined pyrethroid and repellent-impregnated fabrics impregnated by the long-lasting polymer-coating multilayer technique (Faulde and Nehring 2012). In this study, the ester pyrethroid permethrin and the non-ester pyrethroid etofenprox have been cotreated with either DEET or IR3535 at different concentrations which depended on the number of heterogenic, substance-embedding binding layers polymerized. When using the arm-in-cage test against *Ae. aegypti*, 100% probing and biting protection was preserved for 83 weeks with the 5930 mg DEET/m^2 combined with 2139 mg etofenprox/m^2 fabric, for 72 weeks with the 5002 mg DEET and 2349 mg etofenprox/m^2 material, for 63 weeks with the 3590 mg DEET/m^2 and 1208 mg permethrin/m^2 tissue and for 61 weeks with the 4711 mg DEET/m^2 and 702 mg etofenprox/m^2 fabric (Faulde and Nehring 2012). Simultaneously, an up to 75% quicker contact toxicity of repellent pyrethroid fabrics was documented when compared to the corresponding pyrethroid-specific contact toxicity alone using the WHO cone test (WHO 2013) including continuous forced contact of test mosquitoes (Faulde and Nehring 2012; Marius et al. 2017). Obviously, this novel method represents a highly promising approach because (1) insecticide-repellent combinations on fabric are shown to be highly effective against *kdr* and *ace-1R* mutation-containing mosquitoes (Pennetier et al. 2010) and (2) host-seeking mosquitoes come into close contact with the evaporated spatial skin repellent molecules, evidently leading to an acute intoxication due to the insecticidal properties of the skin repellents DEET and IR3535 (Pennetier et al. 2010; Faulde and Nehring 2012). More research work is needed in this area in order to further analyse the effectiveness against mosquito-borne diseases, entomological aspects regarding behavioural, physiological and genetic resistances in vector mosquitoes, epidemiological aspects as well as user safety. Although considered as being very promising, long-lasting insecticide-repellent-treated fabrics, including bed nets and clothing, are not yet commercially available.

12.4 Entomological, Behavioural and Epidemiological Aspects of Insecticide-Treated Textiles

In order to optimize the protective efficacy of insecticide-treated textiles and for the development of synergistic combinations of available personal protective measures against mosquito bites, it is essential to precisely know the species-specific bionomics as well as host finding and feeding behaviour of the relevant mosquito vectors abundant in a given region or ecotope. Since only anthropophilic (species primarily biting man) and anthropozoophilic (species biting humans as well as vertebrate and other animals) mosquito vectors are of special public health concern and importance, less relevant zoophilic disease agent-carrying mosquitoes have been neglected in the context of this study. Furthermore, it is essential to differentiate between indoor (endophilic) biters and those species, which exclusively bite outdoors (exophilic) while never or very rarely entering human dwellings. Additionally, it has to be taken into special consideration whether the vector species of interest are daytime or night-time (nocturnal) biters or follow another circadian rhythm, e.g. biting activities exclusively during dusk, dawn or high noon. In general, *Aedes* mosquitoes are daytime biters, whereas *Culex* and *Anopheles* mosquitoes are primarily feeding during the night-time. Consequently, LLITN use aims at preventing from bites of nocturnal, indoor-biting mosquitoes of the genera *Culex* and *Anopheles* particularly and is, therefore, a key element in roll-back programmes, e.g. against malaria or lymphatic filariosis. Vice versa, LLITN use (a) prevents sleeping healthy humans from infectious bite of nocturnal indoor biters in human dwellings and (b) protects noninfected mosquitoes from indoor infection during blood feeding on sick as well as bedridden persons who contracted a mosquito-borne disease. Unlike LLITNs, insecticide-impregnated clothing protects from mosquito bite at any time at any place and is, therefore, a highly effective means of prevention whenever and wherever the wearer is active outside human dwellings in any urban, rural or sylvatic environment or in buildings while not sleeping under a LLITN.

Like LLITNs, permethrin-impregnated clothing may protect from mosquito bite and can, as well, reduce transmission potential and speed in case asymptomatic viremic human disease reservoirs are exposed to susceptible but not yet infected mosquito vector species. This effect may contribute to a disruption of the transmission chain and is discussed as an option for public health protection (Wylie et al. 2016; Most et al. 2017). Due to a differing genus- and species-specific susceptibility of mosquitoes to insecticides, concentration-dependent permethrin-related excito-repellency, knockdown and kill effects were highest in *Aedes* mosquitoes, followed by *Anopheles* and, finally, *Culex* species (Faulde et al. 2016). Consequently, the protective effect of permethrin-impregnated clothing should be as follows: *Aedes*-borne diseases > *Anopheles*-borne diseases ≫ *Culex*-borne diseases (Faulde et al. 2016).

Interestingly, long-term insecticide exposure may also trigger behavioural changes in mosquitoes which may include change of bionomics, circadian rhythm, sensing of toxic compounds as well as avoiding insecticide-contaminated or insec-

ticide-treated surfaces. In the past, behavioural changes affecting circadian activity and host-finding mechanisms as well as adaption to an altered environment have been well described in anopheline malaria vectors. For example, *An. gambiae* sensu stricto (s.s), one of the most effective African chief vectors of malaria, became more exophilic after introduction of LLITNs in Kenya, whereas it was previously known to be exclusively endophagic (Githeko et al. 1996). Despite its new exophilic behavioural shift, *An. gambiae* s.s. remained highly anthropophilic which increased the overall malaria transmission rate in this area. Following introduction of LLITNs, the most commonly observed effect in *An. funestus* has been a markedly increased exophilic behaviour including a trophic deviation to cattle (Russell et al. 2011). While *An. funestus* populations disappeared in some African regions following introduction of LLITNs (Sokhna et al. 2013), this species developed an entirely new strategy, e.g. in Benin (Moiroux et al. 2012). The formerly nocturnal *An. funestus* strains became more and more daytime biters, as >26% were caught during daylight between 6 a.m. and 9 a.m. (Moiroux et al. 2012). In a more recent study, LLITN use induced a significant behavioural change in anopheline mosquitoes after the third year of use resulting in an activity pattern and host-seeking shift to earlier hours of the evening (Thomsen et al. 2017). Earlier mosquito biting activity during the evening, which happened outside the regular human sleeping period, was linked to an elevated anopheline biting rate outdoors and indoors. As a consequence, LLITN users as well as non-bed net users experienced an increased level of malaria transmission when compared with the conditions prior to the LLITN intervention (Thomsen et al. 2017). In this case, the simultaneous use of permethrin-impregnated clothing could have contributed to enhanced personal protection effectiveness from mosquito bites under the given environmental and epidemiological conditions.

Besides insecticide exposure, anthropogenic environmental changes may also lead to a remarkable chance in mosquito circadian activity and host-seeking behaviour. In India, the usually daytime-active yellow fever mosquito, *Ae. aegypti*, is currently becoming more and more nocturnal while expanding its activity period late into the evening and night-times (ProMED 2017). Obviously, ubiquitous ambient light sources together with higher urban environment-specific night-time temperatures have altered *Ae. aegypti*'s biting habits. Nowadays, dengue, chikungunya and other viral fevers are more frequently transmitted during the night-time outdoors and indoors in this area, making LLITN use more and more effective against *Aedes*-borne diseases (ProMED 2017).

LLITNs, either impregnated with PBO-synergized permethrin or deltamethrin or treated with these pyrethroids alone, do not reduce entry rates of anopheline vector mosquitoes. These results indicate that both synergized and non-synergized pyrethroid-treated textiles do not show a spatial repellent effect due to a missing vapour pressure of this class of contact insecticides (Spitzen et al. 2017). Nevertheless, sublethal toxic effects of pyrethroid-treated bed nets to mosquitoes reduced the number of mosquitoes re-entering the house. Although this insecticide exposure-avoiding mosquito behaviour may be advantageous for LLITN users, malaria transmission may more frequently affect neighbouring, unprotected houses (Spitzen et al. 2017).

12.5 Standardized Efficacy Testing and User Safety

Historically, only military fabrics were impregnated with insecticides by dipping or spraying, but the residual activity was short. In recent years, many armies have developed their own long-lasting impregnated battle dress uniforms using different types of impregnation methods on various kinds of fabric. Although excellent testing guidelines exist for LLITNs (WHO 2013), no World Health Organization Pesticides Evaluation Scheme (WHOPES) or other national or public health guidelines exist for the standardized testing and licencing of insecticide-treated clothing. When taking into account that permethrin-impregnated clothing is widely commercially available in the civilian market since more than a decade, this capability gap strongly deserves consideration in the near future (Faulde et al. 2016).

In order to guarantee that wearing the permethrin-impregnated fabric is protective and safe, both the initial concentration and release rate of permethrin should be monitored through an appropriate quality assurance procedure during the production process, as recommended by the German Federal Institute for Risk Assessments (GFIFRA) (Appel et al. 2008). This is why different internal testing and licencing specifications have been developed among national forces and agencies. In order to ensure that manufacturers fully comply with minimum quality requirements, especially those concerning protective efficacy and user safety, the German Armed Forces (Bundeswehr) implemented the standardized testing and licencing algorithm TL 8305-0331 (WIWeB 2016). Launched in 2002, this standard has been revised to accord with increased technical and scientific knowledge or specific force health protection needs during military deployments. Currently, a first attempt for national standardization of impregnated clothing has been undertaken by the Dutch standardization office by setting up the NEN 8333 “protective clothing—clothing that supports the protection against ticks and is industrially treated with permethrin” (NEN 2017). The national implementation of this norm is planned for early 2018.

In order to analyse the brand-specific characteristics and possible heterogeneity of commercially available permethrin-impregnated clothing on the international market, a selection of widely used products needs to be investigated and compared in detail. In a comparison of the residual bioactivities and laundering resistances of five commercially available, factory-treated permethrin-impregnated fabrics designed for the prevention of mosquito-borne diseases, an extremely high variability in initial permethrin concentrations, residual bioactivity and permethrin loss during laundering was observed (Faulde et al. 2016). The resulting data indicate that only one of the examined products completely met all the necessary efficacy and safety requirements defined by TL 8305-0331 (Faulde et al. 2016). Because of the lack of mandatory international testing and licencing procedures, industrial producers of impregnated clothing generally do not inform on specific impregnation techniques employed, initial insecticide concentrations, arthropod toxicity, residual activity, laundering resistance or durability of the insecticidal ingredient. It was, therefore, interesting to observe that two products (40%) exceeded the initial maximum permethrin concentration of 1300 ± 300 mg/m^2 recommended according to

the current risk assessments for human safety (Appel et al. 2008), showing an extremely high permethrin concentration ≥4000 mg/m^2. One product exhibited a residual permethrin concentration of 1800 mg/m^2 which exceeded the maximally recommended concentration even after 100 standardized machine launderings according to EN ISO6330:2000. When compared with the initial concentrations, the percentage permethrin loss, following 100 launderings was extremely diverse and ranged from 58.14 to 98.46% (Faulde et al. 2016). Chiefly, the higher the binding capacity, or fixation rate, of permethrin fixed onto the fibres was, the lower was the permethrin concentration-dependent bioactivity when investigated against arthropods. Experiences made in this study indicate that a certain impregnation method-related optimal equilibrium has to be identified in order to maximize bioactivity, bioavailability, insecticide stability and protective efficacy of a permethrin-impregnated fabric. Furthermore, the residual permethrin concentration found after 100 launderings was considerably low in two products which showed less than 40 mg/m^2. Too low and potentially sublethal permethrin concentrations on clothing of ≤200 mg/m^2 should be avoided because (a) the corresponding bioactivity falls short of the minimum protective efficacy needed, (b) undesired behavioural changes in arthropod vectors may occur including stimulation or acceleration of attachment of ticks and (c) genetic, physiological or behavioural resistance mechanisms can be triggered (Most et al. 2017).

Depending on the information needed, three main laboratory test systems have been reported for efficacy testing of insecticide-treated textiles: the tunnel test for bed net testing, the cone test and the arm-in-cage test (WHO 2013; DeRaedt Banks et al. 2015; Marius et al. 2017; Most et al. 2017). The use of the tunnel test is recommended by WHO in order to test LLITNs for mortality and blood-feeding success of host-seeking mosquitoes (WHO 2013). As blood host, an animal as bait (usually a guinea pig or a rabbit) is exposed against test mosquitoes in a choice box simulating human bait.

The cone test (Fig. 12.4) measures acute toxicological aspects of test animals exposed for a defined time frame, or continuously, to a contaminated surface. When analysing toxic effects of contact insecticides like pyrethroids, carbamates or organochlorides, direct exposure of test animals is essential. In contrast, substances like organophosphate insecticides as well as arthropod repellents—characterized by a more or less pronounced vapour pressure—affect the health of test animals not only by direct body contact to the treated surface but additionally due to the atmosphere-borne incorporation of molecules which evaporated into the aerosphere. Consequently, forced contact to insecticide-contaminated test surfaces is requisite especially when examined against flying test arthropods like mosquitoes which can actively avoid contact with chemicals (Fig. 12.3b). In case direct exposure of test animals to contaminated surfaces is not carried out continuously, it is absolutely necessary to exactly define the sum of time of exposure required in order to detect the desired insect-specific toxicological actions in detail, like hot-feed, knockdown, excito-repellency or kill effects. As an example, an exact contact exposure time of 3 min is given for test mosquitoes employed in the WHO cone test bioassay set up for standardized LLITN testing (WHO 2013). When compared with *Anopheles* and

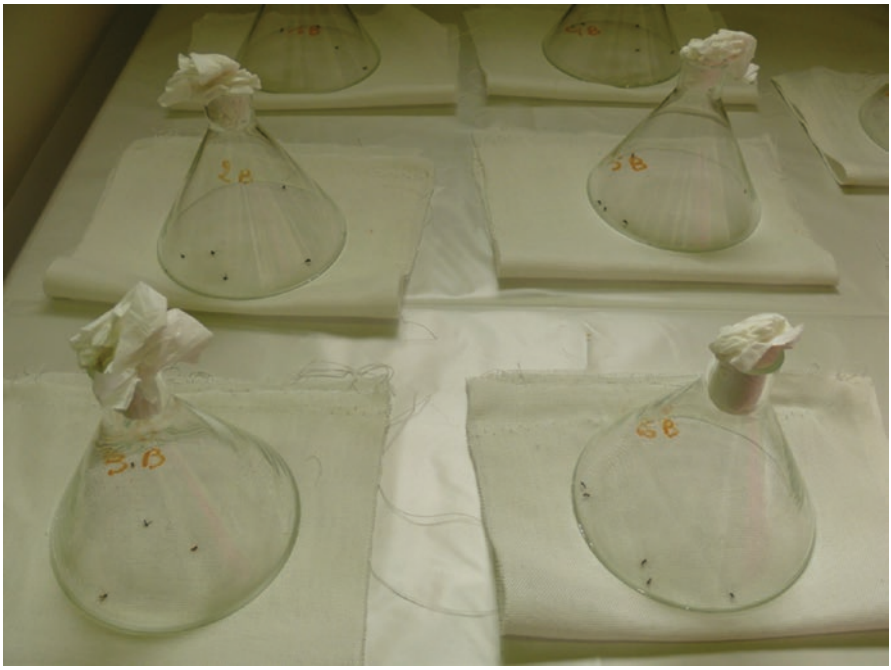

Fig. 12.4 Cone tests employed according to the TL 8305-0331 licencing procedure measuring the insecticidal effects of permethrin bound to laundered and unlaundered fabric against the most pyrethroid-sensitive vector mosquito, *Aedes aegypti*

Culex vector mosquito species recommended for testing purposes, the yellow fever mosquito, *Aedes aegypti*, has been identified as the most suitable biosensor for testing the toxicological effects of pyrethroids on fabrics, especially when monitoring for low doses or cut-off values of less residual impregnation methods (Faulde et al. 2016; Osborne et al. 2016).

The arm-in-cage test has been designed to analyse and monitor the spatial effects of skin repellents (Masetti and Maini 2006). Non-blood-fed test mosquitoes, reared in a cage, were directly exposed to treated (positive control) vs. untreated (negative control) skin of human or animal bait. This system has been also used to measure the spatial repellent effects of textiles impregnated with skin repellents (Faulde et al. 2010). Besides spatial repellency testing, the arm-in-cage test can be modified in order to measure excito-repellency effects, including landing counts, probing activity and blood feeding of pyrethroid-treated textiles (Fig. 12.5) wrapped around the forearm of a human volunteer or around test animals (Faulde et al. 2012; DeRaedt Banks et al. 2015). This test system can also be employed for investigations aiming at further determination whether a clothing fabric is mosquito bite-proof due to mechanical characteristics, including special knitting techniques, internal semipermeable membrane layers or just thickness of the fabric exceeding the length of the mosquito proboscis. For example, in Fig. 12.6, bite-proof combat gloves are quality-tested against *Ae. aegypti* using the arm-in-cage test.

Fig. 12.5 Arm-in-cage test showing excito-repellency including landing, probing and biting behaviour of *Aedes aegypti* on battle dress uniform fabric treated with 1300 mg permethrin/m^2 prior to laundering

Fig. 12.6 Arm-in-cage test showing landing and probing behaviour of *Aedes aegypti* on an untreated but bite-proof battle dress uniform fabric. *Aedes* mosquitoes were not capable to bite through the textile

In order to guarantee user safety, permethrin concentration on clothing is limited to 1250 mg/m^2 according to the US Environmental Protection Agency (Young and Evans 1998; US Armed Forces Pest Management Board 2009) or to 1300 ± 300 mg/m^2 by the GFIFRA (Appel et al. 2008), respectively. GFIFRA performed a toxicological risk assessment following human biomonitoring investigations among users of the German Federal Armed Forces battle dress uniforms (BDUs) as well as on the BDU itself including toxicological studies regarding permethrin concentration, migration rates and exposure criteria (Appel et al. 2008). It has been concluded that normal use of BDUs, impregnated by a defined polymer-coating technique at a concentration of 1300 mg permethrin/m^2 with a 25:75 cis/trans ratio, would not affect human health while ensuring sufficient protection against bites of arthropod vectors (Appel et al. 2008). Corresponding data are not yet available when permethrin treatment has been carried out by spraying or dipping techniques. Additionally, GFIFRA recommended monitoring the maximum permethrin concentration as well as its

binding method-specific release rate through an appropriate quality assurance procedure during the production process in order to assure that wearing impregnated clothing is both protective and safe (Appel et al. 2008). Human biomonitoring studies using Bundeswehr BDUs revealed that permethrin metabolites increased approximately 200-fold during an 8-h workday when compared with the negative control group. When analysed quantitatively, the permethrin content migrated into the human corresponded to roughly 20% of the acceptable daily intake (ADI) of 0.05 mg/kg body weight per day (Appel et al. 2008). During military deployment when wearing impregnated BDU on average 16 h per day, the quantitatively measured permethrin concentration reached approximately 40% of the ADI (Zimmer and Faulde 2009). Consequently, continuous use of newly impregnated BDUs under worst-case scenarios would theoretically lead to a maximum incorporation rate of 60% of the ADI and is, therefore, considered safe. It is yet unclear in how far markedly varying impregnation technique-dependent migration and release rates of permethrin may affect the ADI value during worst-case use, because the permethrin release rate of dipping or spraying methods doubled when compared to the polymer-coating technique and the cross-contamination rate during storage increased 3.5- to 10-fold, respectively (Faulde et al. 2006).

Concurrent with the ongoing Zika virus epidemics in the Americas which showed devastating consequences for foetuses and newborns during prenatal virus infection, the urgent need to optimize personal protection of pregnant women against infected mosquito bite has been identified. Since the synergistic combination of skin repellents together with permethrin-impregnated clothing has been considered as most suitable option, the safety and toxicity of DEET and permethrin have been re-evaluated, especially for its combined use during pregnancy (Wylie et al. 2016). Results obtained led to the strong recommendation that pregnant women should treat their clothing with registered products containing the excito-repellent permethrin which should not be applied directly to the skin (Wylie et al. 2016). Furthermore, the WHO considers permethrin use as being compatible with breastfeeding (WHO 2002). Additionally, a new study published by GFIFRA documented that allergies, linked to sensitization of users by permethrin which has been impregnated on textiles, are highly unlikely (BfR 2017). It has been, furthermore, determined that the systemic cancerogenic effect of permethrin-treated textile use is negligible (BfR 2017).

12.6 Conclusions

Besides chemoprophylactic regimens, personal protective measures against mosquito bites, especially the use of long-lasting factory-treated permethrin-impregnated clothing, may prevent both individual infection as well as the further spread of mosquito-borne diseases of public health concern. Furthermore, it is highly recommended to employ long-lasting impregnation methods exclusively, while aiming at initial maximum permethrin concentrations $\leq 1300 \pm 300$ mg/m^2 and remaining minimum residual permethrin concentrations ≥ 200 mg permethrin/m^2

during field use in order to prevent from possible adverse health effects for users and to ensure sufficient protective efficacy while simultaneously avoiding undesired behavioural side effects and/or pyrethroid resistance in arthropod disease vectors. New and improved strategies for personal protection from bites of hematophagous vector mosquitoes are urgently needed, especially in the light of increasing insecticide resistances including genetic (*kdr*- and *ace-1R*), physiological and behavioural resistance mechanisms. One new attempt, the long-lasting textiles containing a combination of spatial skin repellents, characterized by spatial insecticidal properties, together with excito-repellent and safe contact insecticides, like the pyrethroid permethrin, definitely deserve special consideration and future research work.

In concordance with the EU Biocides Regulation 528/2012, it is strongly recommended that manufacturers of impregnated clothing provide data on concentrations, migration rates, homogeneity on impregnated fabrics, protective efficacy and laundering resistance of the insecticide used for their products. This information is critical to designing safe and effective personal protection products which especially ensures secure use for children and during pregnancy. Although the polymer-coating method has been shown to provide excellent laundering resistance, residual stability and long-term efficacy, current experiences made point out the need for further improvements in residual permethrin-binding techniques and bioavailability (speed of toxic action depending on molecule diffusion processes), especially when garments are intended for long-term worst-case field use.

Long-lasting factory-based polymer-coated permethrin-impregnated clothing provided excellent protection against bites of infectious anopheline mosquitoes, thereby reducing malaria incidence rates significantly in high-transmission foci. Due to a documented higher susceptibility of *Aedes* mosquitoes to permethrin, it can be further concluded that the protection rates against *Ae. aegypti*- or *Ae. albopictus*-borne diseases are equivalent or even higher. In the light of available research data, long-lasting permethrin-impregnated clothing is highly recommended for personal protection against mosquito-borne diseases of public health importance, including chikungunya, dengue, West Nile and Zika fevers, all of which are currently resurging globally.

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Chapter 13
Molecular Aspects of Species of the Genus *Aedes* with Epidemiological Importance

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Abstract Some species of the genus *Aedes* stand out as vectors of several major viruses that affect the world by transmitting serious diseases, including malaria, dengue, yellow fever, Zika, and chikungunya. With the dissemination of arboviruses, there is a constant search for new methods to control such diseases and their vectors. In this regard, the fields of genetics and molecular biology have presented promising alternatives while clarifying questions on the transmittance potential of vectors and the differences among species that arise from their genetic variability. Knowledge of intraspecific and interspecific differences may provide tools to devise

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new insecticides and to elucidate factors that determine resistance against available insecticides. This chapter deals with molecular aspects of the species of the genus *Aedes* that has immense epidemiological importance; we have addressed subjects, including the genome, vector competence of *Aedes aegypti*, and genetic variability of species of this genus detected by the molecular markers; in addition, genetic controls, which consist of dissemination of factors or genes that reduce the propagation of the virus transmitted by the insect through mating or genetic inheritance, have also been addressed in this chapter.

Keywords Mosquitoes · *Aedes* · Genetics · Molecular markers · Public health

13.1 Introduction

Mosquitoes function as vectors of some major viruses that affect the world, which makes them significant as transmitters of serious diseases, such as malaria, dengue, yellow fever, Zika, and chikungunya. This presents a challenge to health services all over the globe to not only generate but increase costs to be able to combat these vectors and treat patients. It is estimated that in 2013 only, about 3.2 million severe cases of dengue were reported with 9000 deaths occurring in medium and low-income countries (Stanaway et al. 2016). It is important to note that the data collected or omitted from reports are underestimated, i.e., there can be a mismatch between the reported estimations and the actual number of cases; studies have indicated that about 70% of infected patients choose not to seek treatment (Bhatt et al. 2013).

According to the World Health Organization, dengue is an endemic in more than 100 countries, including the areas of Africa, Eastern Mediterranean, Americas, Southeast Asia, and the Western Pacific, with the last three regions being the most affected (WHO 2016). Although dengue is treated as the main arbovirus, it may also be related to the genus *Aedes* infections, such as yellow fever, chikungunya, and Zika fever.

Yellow fever is potentially fatal; it is an endemic in the Americas and Africa and has already devastated populations in South America, Central America, and North America. It reached Europe in the eighteenth and nineteenth centuries, but its threat was circumvented with the onset of a vaccine against it. In the health context, dengue became the most important arbovirus with high mortality rates, mainly due to its hemorrhagic potential (Gubler 2011; WHO 2013). Its incidence has increased considerably since with about 390 million cases reported per year and 96 million cases presenting its clinical manifestations. It is estimated that about 3.9 billion people are at a risk of infection in at least 128 countries (Bhatt et al. 2013; WHO 2016).

Chikungunya has already reached more than 2.5 million people in the last 10 years and has been causing major outbreaks (Staples and Fischer 2014). Between

72 and 97% of infected people develop symptoms, such as fever, polyarthralgia, joint pain, arthritis, myalgia, and headaches, with severe cases for heart patients, hypertensives, diabetics, and people over the age of 65 years or neonates (Staples et al. 2009; WHO 2011). The multitude of problems caused by chikungunya may last for years and present a clinical picture similar to the symptoms of rheumatoid arthritis (Bouquillard et al. 2018).

Moving on, also with its many symptoms, the Zika virus infection is an even more serious threat because it is associated with Guillain-Barré syndrome and serious congenital malformations, such as microcephaly (Cao-Lormeau et al. 2016). According to Golding et al. (2015), diseases that have mosquito as a vector correspond to more than 10% of the infectious diseases caused worldwide.

The genus *Aedes* is represented by more than 950 species of mosquitoes. Some are vectors of serious diseases that can lead to death in both humans and other animals. The species are most commonly found in temperate and tropical environments, but some have been introduced in areas of the Americas, Africa, and Asia, facilitating the proliferation of diseases, such as yellow fever, dengue, Zika, and chikungunya (Enciclopédia Britânica 2017).

The main subgenera of *Aedes* are *Ochlerotatus*, *Stegomyia*, *Howardina*, and *Protomacleaya* (Consoli and Oliveira 1994). The *Aedes* species of epidemiological importance are *Ae. aegypti*, *Ae. albopictus*, *Ae. cretinus* (Becker et al. 2010), *Ae. galloisi*, *Ae. flavopictus*, and *Ae. sibiricus* (Schaffner and Mathis 2014).

The World Health Organization advocates combatting the vector by mechanical, physical, chemical, or biological means, which are the main forms of control explored. However, with the dissemination of arboviruses, there is a constant search for new methods to control associated diseases and their specific vectors. In this sense, the studies in genetics and molecular biology have shown promising alternatives as well as clarified questions regarding the potential of these species to act as vectors and have helped understand the differences between them. The genetic control of species is based on the dissemination of factors or genes that reduce pest damage through mating or genetic inheritance. The genetic control of transgenic *Aedes aegypti* type OX513A consists of the production of genetically modified male and female mosquitoes that contain and transfer self-limiting genes. This transgene is lethal to mosquitoes in their larval stage (Harris et al. 2011; Massonnet-Bruneel et al. 2013; Carvalho et al. 2015).

The knowledge in genetics and molecular biology, in addition to gene control, is important to differentiate between species. Studies using molecular biology techniques, such as RAPD, microsatellites, mitochondrial DNA, and isoenzymes, have already demonstrated the existence of several genic types within the same species (*Ae. aegypti)*. To know the intraspecific and interspecific differences is indirectly important for the control of vectors, considering that it can provide tools for the search of new insecticides and also clarify questions related to genetic variability, a determinant factor in the resistance to available insecticides.

13.2 Genome of the Species of the Genus *Aedes*

The mosquito, *Ae. aegypti*, is the world's main vector that carries arboviruses, including dengue, chikungunya, and Zika virus. Therefore, it has been one of the most intensively studied species of arthropods, resulting in the development of detailed genetic and physical maps with considerable insight into the organization of its genome. The research community has developed advanced molecular tools that facilitate the sequencing of the whole genome (Nene et al. 2007). The identified genetic functions may be common to all mosquitoes or unique to an individual species, e.g., the specific behaviors for search of blood to feed as well as the innate immune responses to the pathogens found in the blood (David et al. 2004).

DNA sequencing has the potential to provide new ways for research on insecticide production and genetic alterations to prevent the spread of insect-borne viruses. The institutes J. Craig Venter, European Bioinformatics Institute, Broad Institute, and the University of Notre Dame have published studies on the sequencing of the genome of *Ae. aegypti* in 2007, whereby they identified about 1.38 billion base pairs containing about 15,419 genes encoding mosquito proteins (Barbara et al. 2014).

Studies of these sequences indicate that the species diverged from the fruit fly (*Drosophila melanogaster*) about 250 million years ago, while *Anopheles gambiae* (another mosquito species, whose genome was sequenced) diverged from the fruit fly about 150 million years ago (Kowalski 2007).

13.2.1 Genetic Mapping

A relatively large number of stocks of mutant morphologies have been identified. Isoenzymes provide a tool for the development of the first detailed genetic linkage map for any mosquito species. Many of the stocks of mutant morphologies are still available in individual laboratories around the world and function as valuable tools in investigations associated with other molecular markers and technologies (Fagerberg et al. 2001).

The detailed genetic maps of DNA were constructed using RFLP (restriction fragment length polymorphism) and SSCP (single-stranded conformation polymorphism) loci. The microsatellite markers were not useful or abundant in *Ae. aegypti*, probably due to the organization of the genome. That is, the microsatellites in this species tend to be underrepresented in the genome, and those that are present are often embedded in repetitive elements that prevent their use as single copy markers (David et al. 2004).

However, a 205 cM composite binding map that includes 141 RFLP, SSCP, and SNP loci (single-nucleotide polymorphism) was previously described (Severson et al. 2002). The SNPs appear to be abundant in the genome of *Ae. aegypti*, and because of the potential for high-throughput analysis, they will likely become the marker of choice for large-scale mapping and genotyping (David et al. 2004).

In their studies, Whitfield et al. (2017) reported an understanding of the mechanisms underlying mosquito's role as vectors in order to provide new tools to control the propagation of arboviruses.

Researchers have identified that insects explore two different pathways: interfering RNA (RNAi) [short-interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs)] and transposons to combat viral infection. Endogenous viral elements (EVEs) are non-retroviral virus sequences that are inserted into the mosquito genome and can act as models for the production of piRNAs. The EVEs, therefore, represent a record of past infections and a reservoir of memory with immune potential (Severson et al. 1993).

The large-scale organization of EVEs is difficult to perform with short-read sequencing because they tend to integrate into repetitive regions of the genome. To describe the diversity, organization, and function of EVEs, researchers took advantage of the contiguity associated with the long-read sequence to assemble a high-quality Aag2 cell line genome derived from *Ae. aegypti*, which is an important and widely used model system (Whitfield et al. 2017).

These studies showed that EVEs are acquired through recombination with specific classes of long terminal repeat (LTR) retrotransposons and organized into large loci (>50 kbp) characterized by high-density LTR. The loci containing EVE increased the density of piRNAs as compared to similar regions without EVEs. In addition, it has been detected that piRNAs derived from EVE are consistent with a targeted processing of persistent virus genome infections. In this way, the researchers suggested that comparisons of EVEs in mosquito populations may explain differences in vector competence, and further studies of the structure and function of these elements in the genome of mosquitoes may lead to epidemiological interventions (Morlais and Severson 2003).

In Fig. 15.1, as illustrated by Whitfield et al. (2017), a genome of endogenous viral elements (EVEs) present was characterized in the lineage of cells derived from *Ae. aegypti* Aag2 cell line, using long-read sequences, which are highly repetitive in the mosquito genome. They explored the origin of these sequences and their potential role in mosquito immunity.

13.3 Vector Competence of *Aedes aegypti* and Its Relationship with Genetics

After ingestion by a female mosquito in a blood meal obtained from an infected vertebrate, the pathogen should avoid an arsenal of internal defense mechanisms dedicated to the recognition and subsequent destruction of non-own entities (foreign). The vector competence varies significantly, both within a species and between different species of mosquitoes, in addition to being related to a specific pathogen. It is well documented that this variability is strongly influenced by genetic factors and the control of vector competence is due to the action of two or more individual genes (Barillas-Mury et al. 2000).

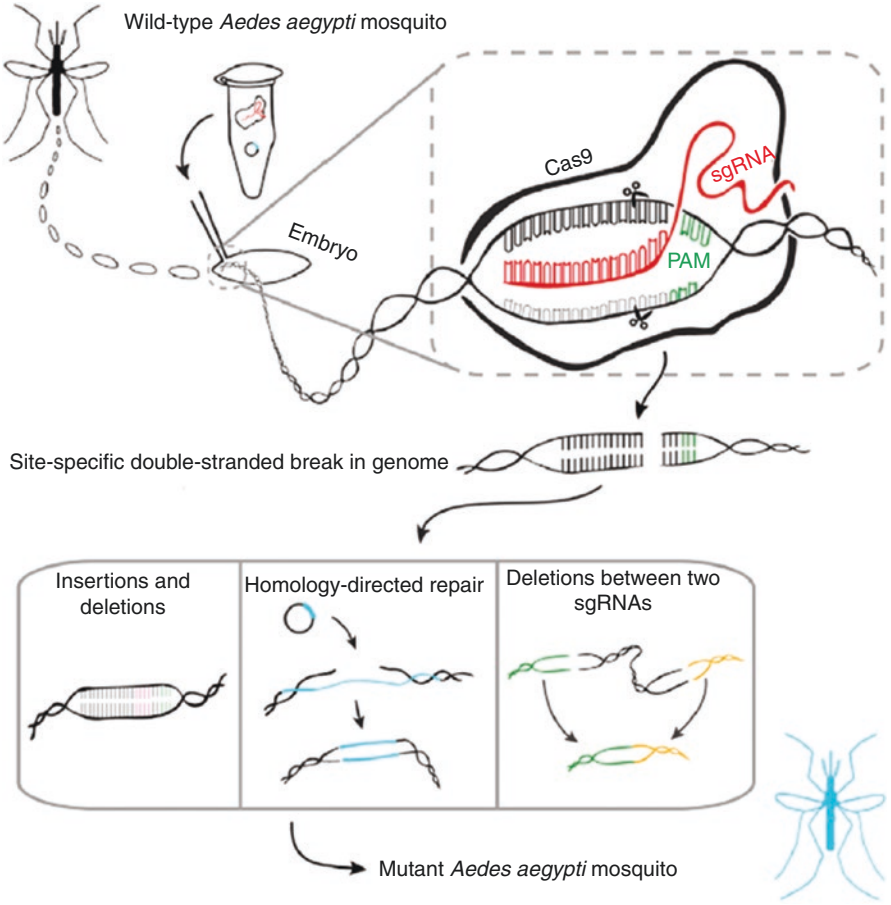

Fig. 15.1 Genome of endogenous viral elements (EVEs) present in cell line derived from *Aedes aegypti*. (Reproduced from Whitfield et al. 2017)

Variable phenotypic traits are results determined by the combined effects of various genes and their interactions with the environment, where vector competence is generally referred to as multigenic characteristics or quantitative traits and the site of an individual gene is called a locus of characteristics quantitative (LCQ). For most quantitative traits, little is known about the number of genes involved, the location of the chromosome, or its gene product (Kowalski 2007).

The development of marker based on DNA loci provides the tools that allow identifying complex traits in their individual genetic characteristics (Lowenberger 2013).

The oral infection of *Ae. aegypti* by the dengue virus has been shown to be variable between geographic strains. Several environmental factors have been shown to affect the extrinsic incubation period (PEI) of arboviruses in mosquitoes, including temperature, humidity, and virus type in humans (Wallis et al. 1985).

The selection experiments confirmed a genetic component in the competence of the flavivirus vector but also showed considerable variability of success in the selection of highly refractory and highly susceptible strains of *Aedes* (Miller and Mitchell 1991). The PEI takes 10–14 days, but in some *Ae. aegypti* populations, it was shown that disruption occurred at the stage, where the virus infected the middle intestinal epithelium or before the dissemination to the same epithelium (Black et al. 2002).

These are commonly referred to as middle intestinal infection barrier and the middle intestinal escape barrier, respectively. Laboratory studies have shown that the competence of the dengue vector in *Ae. aegypti* is a quantitative characteristic. Studies have also documented the existence of genes that determine both middle intestinal infection barrier and the middle intestinal escape barrier (David et al. 2004).

The LCQ mapping studies have confirmed the presence of a dengue vector competence multigene and have also defined genome regions containing three independent LCQs that are involved in both middle intestinal infection barrier and the middle intestinal escape barrier. Two LCQs were reported to be associated with a middle intestinal infection barrier on chromosomes 2 and 3 that represented 44% and 56% of the total phenotypic variance, respectively. A lower LCQ effect for middle intestinal escape barrier was identified on chromosome 3 (Black et al. 2002; David et al. 2004).

13.4 Genetic Variability of Species of the Genus *Aedes*

Molecular techniques have made possible the knowledge of the epidemiological aspects of different diseases. Molecular markers have proved useful to studies of genetic diversity of *Aedes* mosquitoes and evaluation of the structure of their populations. These studies have generated essential information for the understanding of the transmission potential of these vectors, helping to establish infectious diseases prevention and control measures.

13.4.1 Isoenzymes

Sequences of the genes coding for isoenzymes were studied for the characterization of genetic variation among populations of *Ae. aegypti* from distinct provinces of Thailand with many records of dengue cases. Thirty-one loci coding for isoenzymes were analyzed, and 19 loci of these were polymorphic. Low intra- and interpopulation genetic diversity were observed (Sukonthabhirom et al. 2009).

Other genetic variability studies were conducted in Vietnam, concluding that mosquitoes collected on the Commuter Belt and in Ho Chi Minh City Center were significantly and highly differentiated, while gene flow was low (Tien et al. 1999). In Brazil, an evaluation of susceptibility to yellow fever and dengue virus 2 was

performed in *Ae. aegypti*. Among the ten loci examined, only one was monomorphic in most samples studied (Bracco et al. 2007).

Studies with isoenzymes carried out in populations of *Aedes albopictus* from Italy, the United States, Indonesia, and Japan showed that of the 18 loci studied, 15 presented polymorphism and suggested the existence of genetic drift, besides having high genetic similarity between the populations of Italy and the United States, indicating that the introduction of this species in these countries occurred in a similar way (Urbanelli et al. 2000).

13.4.2 Randomly Amplified Polymorphism DNA (RAPD)

The RAPD technique was employed in a study of genetic structure analysis of populations of *Ae. aegypti* from 11 cities, distributed in 6 states of Brazil: Alagoas, Ceará, Mato Grosso do Sul, Paraná, Rondônia, and São Paulo. In populations of the different states and in the samples obtained from cities of the same state, the existence of polymorphism and elevated levels of genetic variation were observed. These data show that populations of *Ae. aegypti* in Brazil present significant genetic differences. Regression analysis of geographic distances and pairwise F_{ST} values determined by RAPD markers indicated that there is a correlation between genetic structure and geographic location (Paduan et al. 2006).

Levels of intraspecific polymorphism and genetic relationships of five populations of *Ae. aegypti* from Argentina (Villa María, Córdoba, Buenos Aires, Orán, and Posadas) were searched by RAPD. The three primers used for DNA amplification produced 17 different bands. The cluster analysis revealed that the population from the city of Córdoba was more genetically related to from Orán and the sample of Buenos Aires had more similarity with the population of Posadas (Sousa et al. 2001).

Ae. albopictus populations of the Brazilian states of Minas Gerais, Pernambuco, and Rio de Janeiro were analyzed by RAPD. Populations of Minas Gerais and Rio de Janeiro presented higher genetic similarity, and there a restricted gene flow was observed among all populations (Ayres et al. 2002).

13.4.3 Microsatellites

A total of 11 microsatellite loci were used to evaluate the genetic alterations of *Ae. aegypti* collected during relatively wet and dry seasons from three localities in the city of Cebu, Philippines. Seasonal variation was observed in allelic frequencies and allelic richness. The mean genetic flux was higher in the rainy season than that in the dry season (Sayson et al. 2015).

The genetic variability of *Aedes taeniorhynchus* was evaluated in Colombia using eight microsatellite DNA loci. Five polymorphic microsatellite loci were

found with 19 alleles showing 62.5% polymorphism. The mean number of alleles per site was 3.8. The mean heterogeneity ranged from 0.568 to 0.660. Most of the polymorphic microsatellite loci were in imbalance due to the deficit and the excess of heterozygotes, which highlighted the genetic homogeneity between these populations. There was no significant linkage disequilibrium between pairs of genotypes from the various populations, but it was possible to conclude that this mosquito is distributed in local populations along the Colombian Atlantic coast (Bello and Becerra 2009).

13.4.4 Mitochondrial DNA (mtDNA)

Paduan and Ribolla (2008) evaluated the variation among 125 specimens of *Ae. aegypti* from different geographic areas, based on cytochrome c oxidase (*COI*) and *ND4* sequences in mitochondrial DNA (mtDNA). The analysis with *COI* and *ND4* revealed the existence of 7 and 24 different haplotypes in the mosquitoes collected, respectively. In the analysis of molecular variance, it was observed that the variability in the genetic structure among populations was expressive.

The mitochondrial *ND4* gene was analyzed in a population genetics study, which evaluated 19 populations of *Ae. aegypti* found in Thailand. A total of seven distinct haplotypes were obtained, and the gene flow among the populations varied considerably. Regarding genetic diversity, this was much lower than what was observed in other studies with *ND4* of *Ae. aegypti* (Bosio et al. 2005).

In Manaus, Brazil, a study of variability and genetic structure of *Ae. albopictus* populations, based on the polymorphism of the mtDNA *ND5* gene, indicated that the two haplotypes were shared with populations of other regions and that there were occurrences of at least two introductions of *Ae. albopictus* in the state: one probably from the South coast and Southeast of Brazil and the other from the coast of Florida in the United States (Maia 2008).

Devicari and Suesdek (2010) identified differences in three *Aedes scapularis* populations in the state of São Paulo (Brazil): two in the metropolitan region at a distance of 20 km from each other and 200 km from the third, using the *COI* genetic marker. The marker showed an even intrapopulational difference demonstrating the sensitivity of the technique.

13.5 Genetic Control of Mosquitoes of the Genus *Aedes*

There are no vaccines available for dengue, chikungunya, or Zika as yet, and this makes vector control the only alternative to minimize their transmission and, thus, to reduce the dissemination of associated diseases (Winskill et al. 2014). However, the procedures used to control disease-bearing mosquito populations have not been as effective for some key species that use small dispersed bodies of water as

breeding sites (Alphey et al. 2013). The cost of permanent public programs (accentuated by the toxicity of insecticides to nontarget organisms), the selection of strains resistant to chemical products, and the high reproductive capacity of these vectors are main factors that hinder their control in a certain locality (Oliveira et al. 2011). In this way, the search for other more efficient methods to contain these vectors has increased. The genetic control of vectors mosquitoes by means of the sterile insect technique (SIT) and release of insects carrying a dominant lethal gene (RIDL) it is among the proposed strategies.

13.5.1 Sterile Insect Technique (SIT)

The sterile insect technique (SIT) is an alternative control tool that involves the production of a large number of insects of the target species, where male insects have been sterilized through exposure to gamma or X-rays. This exposure causes random chromosomal rearrangements; these irradiated organisms are then released into the environment (Shelly et al. 2007; Zara et al. 2016). A large number of sterile individuals should be released in a sustained and systematic manner in the target area so that there is an effective competition with the wild males (Vreysen et al. 2006). From the crossbreeding between sterile males and wild females, unviable eggs are produced (Fig. 15.2), and thus, the reproductive potential of females is reduced (Shelly et al. 2007). The ultimate result is a decrease in the population of vectors.

The SIT has been used over the last 50 years and is currently carried out on all continents (FAO/IAEA 2017). The first application against insects was in the program that successfully eradicated the pest species, *Cochliomyia hominivorax*, extensively from areas of North and Central America. This fly, commonly known as

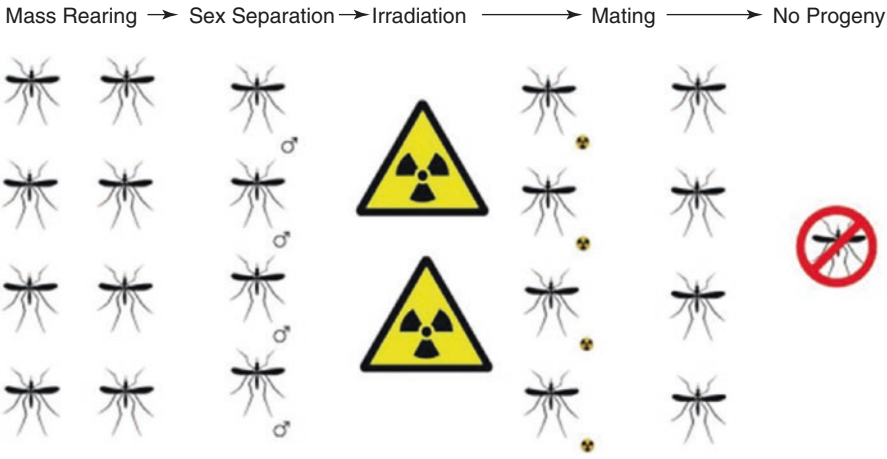

Fig. 15.2 Stages of the conventional SIT technique. (Reproduced from Wilke and Marrelli 2012)

the New World screwworm (NWS), is an agent of myiasis in humans and other warm-blooded animals and is a pest with economic importance in agricultural systems that have large cattle production (Hendrichs 2017).

With regard to studies that evaluated the use of SIT for the control of vectors of the genus *Aedes*, Oliva et al. (2012) found that sterile males of *Ae. albopictus* could compete satisfactorily to copulate with non-irradiated females, thus contributing to the elimination of the wild population of *Ae. albopictus* on the Reunion Island. When investigating the influence of the release of male *Ae. aegypti* mosquitoes that were sterilized by 0.6% thiotepa on the reproductive potential of populations of this species under confined conditions, Gato et al. (2014) observed that the females' fecundity was considerably reduced, indicating the possible efficiency of the sterile insects in reducing reproductive capacity of the target population and their contribution to vector control.

Bellini et al. (2013) carried out field studies in three urban areas of Italy with the objective of developing a more viable and economical method of applying SIT to combat *Ae. albopictus* populations. Five tests were performed from 2005 to 2009, and it was verified that sterile males were released at a rate of 896–1590 males/ha/week, which promoted sterility in the target population at an expressive level.

A mathematical model was formulated to evaluate the impacts of the entry of sterile male mosquitoes in a previously infested territory, considering the logistic recruitment of these insects. The model showed that the use of other measures that aimed at population reduction of vectors before spraying is fundamental to the efficiency of SIT (Esteva and Yang 2006).

The success of SIT application depends on the sterilization capacity and sufficient distribution of the male insects so that they reach a significant proportion of overflooding in the environment and successfully compete with the wild males in copulating with females in the field. The knowledge of mosquito biology is also important for the programs to optimize their methods and to prevent artifices that may promote the infeasibility or ineffectiveness of SIT (Lance and Mcinnis 2005).

The use of SIT in the control of mosquitoes that are important for public health is advantageous as compared to the use of synthetic insecticides because it ensures the release of environmentally harmless sterile insects that do not present toxic residues nor cause undesirable problems. This strategy controls a particular species with specific measures that do not reach other species (Alphey et al. 2010; Alphey and Bonsall 2017).

13.5.2 Release of Insects Carrying a Dominant Lethal Gene (RIDL)

The RIDL technique was developed by Thomas et al. (2000) based on the SIT method and consists of a species-specific control strategy (Dickens et al. 2016) that produces mass insects carrying a lethal gene with the subsequent release of transgenic males into the environment. The heterozygous progeny, resulting from the

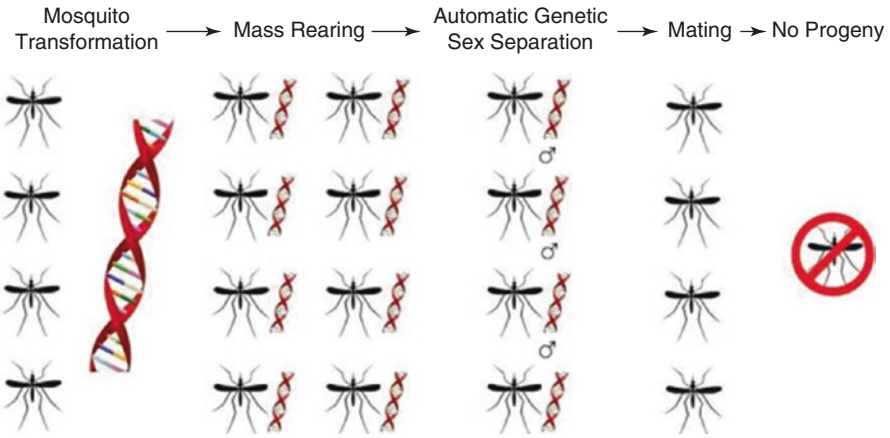

Fig. 15.3 Schematic representation of the operation of the RIDL system. (Reproduced from Wilke and Marrelli 2012)

crossing of transgenic homozygous male insects with wild females, receives the lethal gene and ends up dying prematurely (Alphey and Andreasen 2002) as there is no repressor of the lethal system in its natural environment (Araújo et al. 2015; Fig. 15.3). With the death of mosquitoes carrying the transgene, there is a population reduction of these vectors and, consequently, a decrease in the number of cases of diseases that are transmitted (Oliveira et al. 2011).

The dominant lethal gene is associated with a female-specific promoter, and its expression is deactivated in the presence of the tetracycline suppressor, allowing the survival of the insects (Wilke et al. 2009; Nordin et al. 2013). In order to separate male mosquitoes from females in the RIDL system, tetracycline is withdrawn which promotes the death of females (Wilke and Marrelli 2012).

According to Alphey (2002), a key factor in the RIDL technique is the expression of the tetracycline-repressible transcription activator protein (tTA). This protein is controlled by a selected promoter, and in the absence of tetracycline, binds to a specific DNA sequence (tetO) while activating the transcription of an adjacent minimal promoter, which induces expression of the effector or lethal gene (Fig. 15.4). When there are low concentrations of tetracycline in the system, there is no binding of the tTA protein to DNA or expression of the lethal gene, since tTA prefers to bind to tetracycline rather than to tetO (Oliveira et al. 2011).

The first field study with genetically modified *Ae. aegypti* was conducted in the year 2009 on an area of 10 hectares of Grand Cayman Island. The transgenic male insects were released for a period of 4 weeks, and their crossing with wild females was successfully carried out (Harris et al. 2011).

Evaluation of experimental releases of transgenic mosquitoes has been carried out in countries, such as Brazil, Cayman Islands (UK), France, Guatemala, India, Malaysia, Mexico, Panama, Philippines, Singapore, Thailand, the United States, and Vietnam (Reeves et al. 2012). Carvalho et al. (2015) carried out sustained releases of *Ae. aegypti* modified males for more than a year in the city of Juazeiro,

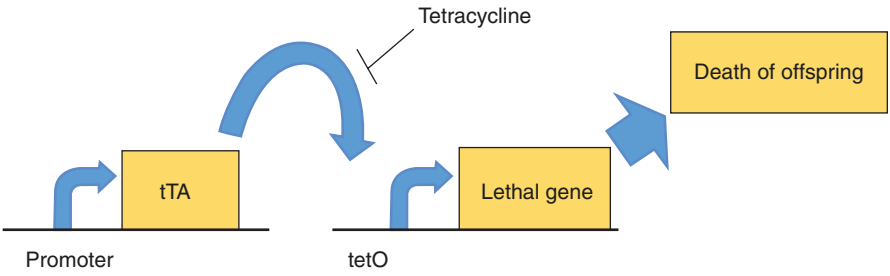

Fig. 15.4 Mechanism of the tetracycline-repressible system. A preselected promoter controls tTA. This protein, when expressed, binds to tetO and leads to the expression of an adjacent minimal promoter, which induces expression of the lethal gene under its control. The expressed lethal gene causes offspring death. (Adapted from Alphey 2002)

Bahia, Brazil. They managed to reduce the wild population of this species by 95%. Garziera et al. (2017) evaluated the disruptive effect of the excessive release of transgenic mosquitoes on the natural population of *Ae. aegypti* in two municipalities located in the Brazilian semiarid region: Jacobina and Juazeiro. The population suppression of mosquitoes in Juazeiro lasted 17 weeks after the discontinuation of the release, whereas the population in Jacobina remained suppressed for 32 weeks. The results of this study indicated that a constant release is required in the treated areas and that, after population suppression, the release may be reduced and employed to prevent external migration.

The easy maintenance of the colony, possibility of separating males from females, less costly production, and high efficiency of the RIDL technique as compared to other mosquito control strategies indicate that it may contribute as an alternative system for the control of agricultural pests and insects of epidemiological importance (Wilke et al. 2009). However, gene silencing or eventual genetic changes that change the purpose of the transgene may occur in RIDL strains (Carvalho et al. 2014). Another limiting factor in the development of RIDL is the need for genomic data (Lin and Wang 2015). Therefore, further studies should be performed to ensure both the improvement of RIDL and its success in controlling vectors of medical interest.

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Chapter 14
Mosquitoes, *Plasmodium* Parasites, and Cancer: Where from, Where to?

Martin Ward and Giovanni Benelli

Abstract *Plasmodium* spp. are among the principal parasites carried by mosquitoes (Diptera: Culicidae), and mosquitoes are probably man's worst enemy. Over thousands of years and across the globe, mosquito-borne diseases have killed, or maimed, humans, other mammals, birds, and reptiles. *Plasmodium* parasites are not exclusive to mosquitoes. It has been reported that *Culicoides* (Diptera: Ceratopogonidae) are susceptible to *Plasmodium* infection, with some of their abdomens containing human blood and infected with avian *Plasmodium*, illustrating how little we know about numerous vectors affecting humans. Furthermore, it has been also outlined that an avian parasite (*Plasmodium lophurae*) can be experimentally adapted to a human host. Mosquitoes also carry other parasites, for example, *Dirofilaria* and hookworms. This chapter, however, will remain focused on the interrelationship between *Plasmodium* spp. parasites and mosquitoes and looks to the future. Will we, as some are beginning to suggest, find that mosquitoes have an involvement in the development of some cancers, and will we find that mosquitoes play a greater role in Lyme disease than hitherto imagined? The chapter is broken into eight sections, the first being an overview of the history of *Plasmodium* and some key facts about mosquitoes, the second looks at the role of mosquito saliva in immunosuppression and the IARC group 1 carcinogens carried by mosquitoes, the third looks at other infectious agents linked to cancer, the fourth looks at current beliefs on cancer causation, the fifth considers how the immune system reacts to these carcinogens, the sixth why the possible role of mosquitoes in cancer development has not been uncovered so far, and the seventh casts a critical eye on developments in mosquito control. Finally, we provide outlooks for future research.

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14.1 A Brief History of *Plasmodium* and Mosquitoes

Plasmodium and mosquito vectors became widely recognized when Ross and Grassi competed to discover the cause of millions of deaths due to malaria (Spielman and D'Antonio 2001; Benelli and Beier 2017). Mosquitoes have been on Earth, and taking blood meals, for at least 46 million years. They have since been found responsible for numerous other insidious human diseases, as well as diseases affecting livestock, pets, and wildlife (Crans 2004; Ferguson et al. 2010; Ward and Benelli 2017a). Murugan et al. (2016) provide a comprehensive list of the main pathogens and parasites associated with mosquito vectors, which according to data from WHO infect more than one million people per year. The history of these diseases is well documented in the literature and does not need to be repeated here. What is interesting, and still inadequately investigated, is the mosquito's possible relationship to other serious diseases currently afflicting mankind, particularly cancer and the growing public health problem of Lyme disease. Indeed, the International Agency for Cancer Research (IARC) has concluded that infection with *P. falciparum* in holoendemic areas is "probably carcinogenic to humans" and placed it in "group 2A" (Bouvard et al. 2012; Benelli et al. 2016a).

The origin of human *Plasmodium* has been debated extensively, being attributed at different times to both avian and simian sources (Ward and Benelli 2017a), with hypotheses that *Plasmodium* spp. switch from birds to African great apes and vice versa (Waters et al. 1991; Cox 2010; Prugnolle et al. 2011; Pick et al. 2011; Perkins 2014; Molina-Cruz et al. 2016). Although regarded as unusual, *Culiseta melanura* can transport malaria parasites from a bird reservoir to the human bloodstream (Spielman and D'Antonio 2001). Furthermore, with human malaria, there are numerous slightly different strains, such that travellers from one district may have immunity against their local strain but be infected as soon as they encounter a neighboring strain (Spielman and D'Antonio 2001). Our overall knowledge of human malaria is still expanding; as recently as 2010, it was proposed that *Plasmodium ovale* comprises two non-recombining species and that morbidity caused by it has been underestimated (Sutherland et al. 2010). For some time, only four *Plasmodium* species have been associated with human malaria, namely, *P. malariae*, *P. vivax*, *P. ovale*, and *P. falciparum*, but in this century, the simian *P. knowlesi* has been added, and very rare malarial cases have been linked with *P. cynomolgi.* In addition, there is possible transmission to humans of *P. cynomolgi bastianelli*, *P. inui*, *P. rodiani*, *P. schwetzi*, *P. semiovale*, *P. simium*, and *P. eylesi* (Ta et al. 2014).

Worldwide there are over 3000 species of mosquitoes (Crans 2004), so it would seem quite possible that further forms of *Plasmodium* could exist in humans, and remain unidentified, particularly if the human host remains asymptomatic. Tests for avian and simian malaria are constantly evolving (Richard et al. 2002; Ta et al. 2014). More recently loop-mediated isothermal amplification tests have increased

accuracy and reduced testing costs and time for human malaria diagnosis (Bousema and Drakely 2011). It has recently been shown that tests for avian *Plasmodium* may have produced false negatives in birds due to different elution protocols (Niebuhr and Blasco-Costa 2016), and parasite genetic information obtained from cultures is likely to be different from the natural infection parasites (Yeda et al. 2016).

General PCR protocols may favor detection of the parasite with the higher parasitemia, and sensitivity of different PCR assays in detection of mixed infections has been insufficiently tested (Zehtindjiev et al. 2012; Bernotiene et al. 2016). It would be realistic, therefore, to assume any tests for the presence of avian *Plasmodium* in humans may have produced false negatives for the same reason. However, a Medline search for humans actually being tested for avian *Plasmodium* failed to produce any evidence. This is perhaps not surprising as over 50 species of avian *Plasmodium* have been identified, and diversity is greater than realized (Njabo et al. 2009; Outlaw et al. 2016). *P. knowlesi* and *P. cynomolgi*, both simian, have been detected in humans and are commonly misidentified (Vythilingam et al. 2008; Cox-Singh et al. 2008; Ta et al. 2014). Huff (1951) records how *P. gallinaceum* and *P. relictum* could go through pre-erythrocytic stages in ducks, geese, canaries, and domestic pigeons without parasitemia. As the number of avian *Plasmodium* is substantial, it would require such a huge study that it would be hard to justify on both cost and ethical grounds if humans were themselves to be tested for each type.

Zoonoses are believed to account for up to 60% of emerging infectious diseases and 71.8% of these originate in wildlife (Jones et al. 2008; Benelli and Duggan 2018). This suggests that any infectious agent that affects primates or birds has a reasonable chance of affecting humans. The World Bank has estimated that zoonoses have cost global economies more than $20 BN in direct and $200 BN in indirect costs between 2000 and 2010. Besides, zoonoses provide opportunities for host switching or genetic exchange giving rise to novel genetic combinations (Webster et al. 2016). Mosquitoes are well placed to transfer zoonoses to humans. In the case of human malaria, dengue fever, chikungunya, and Zika virus, several mosquito species are potential major vectors (Benelli and Romano 2017), raising the prospect that any simian or avian *Plasmodium* that could affect humans may be carried by a vector not previously suspected. Based on laboratory studies, it has been shown that *Aedes albopictus* is a competent vector for at least 22 arboviruses (Malcolm 2009). In addition, it is known that some mosquito species can carry more than one type of *Plasmodium* (Alavi et al. 2003; Perez-Tris and Bensch 2005). Avian malaria parasites are present in numerous mosquito species, for example, *Culex*, *Aedes*, *Culiseta*, *Anopheles*, *Mansonia*, *Aedeomya*, and *Coquillettidia*; the "parasite-vector-vertebrate-host" interactions remain understudied, as do reasons for the pathogenicity of different species; and few experts have the knowledge base to identify avian *Plasmodium* spp. (Braga et al. 2011; Inci et al. 2012). Furthermore, host-parasite interactions are complex, and host and vector ecology may be important in parasite diversification (Lauron et al. 2014). Hybrids may also occur (Ramiro et al. 2015), and to identify *Plasmodium* species, all the main blood stages of the parasite should be examined, with samples containing relatively intensive parasitemia (Braga et al.

2011). In addition to malaria parasites, there are numerous tests for arbovirus recognition in mosquitoes (Anderson et al. 2010).

14.2 A Focus on Lyme Disease and Mosquitoes

Lyme disease, which is normally regarded as a disease transmitted by *Ixodes* ticks (Acari: Ixodidae), may arise more commonly from a mosquito bite. Research shows that the key bacteria associated with Lyme disease, namely *Borrelia burgdorferi*, are present in mosquitoes (Zákovská et al. 2002; Anderson et al. 2006), and mosquitoes from four different genera, *Aedes*, *Culiseta*, *Culex*, and *Ochlerotatus* also carry *Borrelia afzelii*, *B. bavariensi*, and *B. garinii* (Melaun et al. 2016). The US Centers for Disease Control and Prevention estimates that Lyme disease infects 329,000 people annually in the USA and has become one of the most common infectious diseases. Other countries, such as Germany, also suffer many Lyme disease cases per year. The proportion of these cases potentially arising from mosquito biting activity, as opposed to ticks, is unknown.

However, those persons acquiring it from mosquitoes may not incur the typical bulls-eye rash associated with a tick bite, as only around 25% of Lyme patients experience one. A biofilm in Lyme disease has been discovered that boosts therapy resistance by nearly 1000 times (Sapi et al. 2016). Furthermore, patients acquiring Lyme disease from mosquitoes may experience more chronic forms, due to the numerous other pathogens that are present in mosquitoes. Further research on this is urgently needed.

14.3 Mosquito's Saliva and Carcinogenic Agents

It has been found that mosquito saliva has the capacity to activate dermal mast cells and to induce local inflammatory cell influx (Depinay et al. 2006). Mosquito bites consistently induced macrophage inflammatory protein 2 (MIP-2) in the skin and interleukin-10 (IL-10), draining lymph nodes and downregulating antigen-specific T-cell responses by a mechanism dependent on mast cells and mediated by IL-10. *Plasmodium berghei* sporozoites vectored in mice via mosquito bites were more infectious than when sporozoites were injected intravenously, which suggests that saliva plays a role in parasite transmission. Compared with arbovirus infection initiated in the absence of the mosquito or its saliva, infection via mosquito saliva leads to an increase in virus transmission, host susceptibility, viremia, disease progression, and mortality (Schneider and Higgs 2008). Mice infected with West Nile virus (WNV) through the bite of a single infected *Culex tarsalis* mosquito exhibited five- to tenfold-higher viremia and tissue titers at both 24 and 48 h postinoculation and faster neuro-invasion than mice given a median mosquito-inoculated dose of WNV by needle (Styer et al. 2011). The inoculation of mice with WNV

mixed with salivary gland extract gave rise to higher viremia, illustrating that mosquito saliva is the principal cause of mosquito-induced enhancement.

With respect to dengue virus, it has been stated that serine protease activity in *Aedes aegypti* saliva augmented virus infectivity and that mosquito saliva contains a potent mixture of secreted molecules that can affect vascular constriction, blood coagulation, platelet aggregation, inflammation, immunity, and angiogenesis (Conway et al. 2014). It has also been found that the host immune response to mosquito-transmitted chikungunya virus varies from that obtained by needle-inoculated virus (Thangamani et al. 2010). Compared to expression with Sindbis virus alone, IL-10 expression showed a 7.6-fold increase by 72 h postinoculation in mice receiving mosquito salivary gland extract with the virus (Schneider et al. 2004). Any differences in saliva contents between species may affect the transmission of disease, and successful viruses co-evolve with mosquito vectors (Colpitts et al. 2012). Several types of viral pathogens can cause disease in mosquitoes themselves and thus may be passed on to humans. The baculoviruses and cytoplasmic polyhedrosis viruses (Reoviridae; Cyprovirus) are two of the major types. Other significant types of viruses are the densoviruses and the iridoviruses. Cypoviruses are RNA viruses, while others are DNA viruses (Becnel and White 2007).

There are other important observations about mosquitoes. WNV has been isolated from male mosquitoes, which suggests that mosquitoes can become infected by means other than by blood feeding, possibly by transovarial transmission (Anderson et al. 2006). It has also been observed that the dengue structure differs at the temperatures of its human and mosquito hosts (Zhang et al. 2013). This adds additional complexity to the understanding of how a variety of host species contribute to transmission and human infection (Webster et al. 2016).

Immunosuppression is frequently associated with the development of cancer (Hancock 1985; Ciliao et al. 2016), and hence this disease, for which it is known that there are 200 types, may have a relationship to mosquitoes. Numerous diseases are transmitted by mosquitoes. They are recognized for their ability to transmit deadly and debilitating pathogens and parasites, such as filariasis, malaria, WNV, chikungunya, dengue, and Zika virus (Benelli and Mehlhorn 2016). However, there are many less well-known pathogens carried by mosquitoes, for example, 83 bacterial species belonging to 31 bacterial genera in the midgut of *Culex quinquefasciatus* mosquitoes (Chandel et al. 2013). The species all belong to three phyla, *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. A high diversity of microbes was observed in all their samples, and they state that a fraction of mosquito midgut inhabitants could be common for different mosquito species.

The IARC estimates that 20% of cancers worldwide are attributable to infectious agents. It identifies six such agents as causal, one as increasing the risk of cancer from co-infection and one that suppresses the immune system. In addition to these six agents, three parasites have been identified as causal, two of which are mainly limited to the Far East. The causal agents (group 1 carcinogens) are human papilloma virus, hepatitis B and C viruses, Epstein-Barr virus, Kaposi sarcoma herpes virus, human T-cell lymphotropic virus type 1, the bacterium *Helicobacter pylori*, the helminth *Schistosoma haematobium*, and, in Far Eastern cancers, *Opisthorchis*

viverrini and *Clonorchis sinensis*. It is also pointed out that Epstein-Barr virus (linked with Burkitt's lymphoma and malaria, see Lednicky and Butel 1999) is transmitted by mosquitoes and that the human T-cell lymphotropic virus can be transmitted by insects.

The Kaposi sarcoma herpes virus is from the same family of viruses as the Epstein-Barr virus, so it is reasonable to assume that this virus may be capable of being transmitted by mosquitoes. The IARC hepatitis B monograph (100B 2012) states that chronic hepatitis B virus (HPB) infection is responsible for over 50% of hepatocellular carcinomas (one of the most common cancers). HPB is highly contagious and is transmitted by percutaneous exposure to infected blood, where the highest concentration of the virus occurs. As mosquitoes take blood meals, it is reasonable to suppose that they may play a pivotal role in its transmission. Hepatitis C (HPC) is also transmitted by percutaneous exposure to blood. The IARC states that dual infection with both HPB and HPC is common, and several cross-sectional studies have detected the presence of *H. pylori* in HCV-infected hepatocellular cancers, which illustrates the potential for several infectious agents to be acting in unison. Just two ingredients can work synergistically to produce different effects, (HCMV) IE1 and IE2 gene products cooperate with adenovirus E1A gene to transform primary baby rat kidney cells, and the IE1 and IE2 are present only transiently (Shen et al. 1997).

The IARC also reports cases of cross infection with human T-cell lymphotropic virus. We suggest that mosquitoes may be a common denominator in these infections. The IARC has recently stated that cytomegalomavirus (also a member of the same herpes family as Epstein-Barr) may be carcinogenic and malaria, which is of course transmitted by mosquitoes, is a probable carcinogen. The remaining virus recognized by the IARC as causal in cancers is the human papillomavirus. Bovine papillomavirus is suspected of being transmitted by insects so why not human papillomavirus? Besides, there is some present evidence linking cancers with mosquitoes. Hamster reticulum cell sarcoma can be transmitted through the bites of *A. aegypti* females (Banfield et al. 1966).

14.4 Other Infectious Agents Linked to Cancer and Mosquitoes

Aside from the IARC-recognized infectious agents, other infectious agents have been linked to cancer, for example, *Mycoplasma*, *Salmonella typhi*, *Mycobacterium tuberculosis*, *Streptococcus* spp., *Adenovirus*, BK and JC viruses, and *molluscum contagiosum* (Alibek et al. 2013; Samaras et al. 2010; Magar 2006; Sasaki et al. 1995: Sevik 2012). We would stress that while these are important findings, they should not at the present time be given the same weight as IARC group 1 infectious agents.

Mycoplasmas have been associated with human carcinomas with 56% of gastric carcinomas positive for *Mycoplasma hyorhinis* and mycoplasma infection in esophageal, lung, and breast cancers being 50.9%, 52.6%, and 39.7%, respectively (Huang

et al. 2001). Furthermore, mycoplasmas cause haemolytic anemia, and it is known that mosquitoes can transmit the infection (Neimark et al. 2004).

Microorganisms isolated from head and neck tumors include *Veillonella*, *Bifidobacteria*, *Lactobacillus*, *Actinomycetes*, *Microplasma*, and Torque Teno Midi Virus, while others have been isolated from oral squamous cell carcinomas, i.e., *Micrococcus*, *Propionibacterium*, *Bacillus*, *Streptomyces*, *Enterococcus*, *Staphylococcus*, *Pseudomonas*, and *Exiguobacterium* plus several novel species (Alibek et al. 2013; Salmanizadeh et al. 2011; Hooper et al. 2006). MC polyomavirus is found in a high proportion of cancer cases "linearized and clonally integrated in the host cell genome" (Feltcamp et al. 2013). Simian virus 40 has for many years been linked in some way with cancer, and other polyomaviruses are known to cause severe illness in immunocompromised hosts (Feltcamp et al. 2013; White et al. 2005; Rivera et al. 2008; Scuda et al. 2011).

Helminths have been linked with mosquitoes, although, as yet, evidence for a direct link with those pathogens listed by the IARC is lacking (Chene 2009). Torque Teno Midi Virus has been discovered in both neck cancers and in the mosquito midgut (Salmanizadeh et al. 2011). An indirect link has been found between mosquitoes and human T-cell lymphotropic virus (Brooker et al. 2007; Chang et al. 2001; Fouche et al. 1990). There are many unknowns with respect to viruses in mosquitoes, although there is a wide diversity of phage sequences with *Propionibacterium*, *Enterobacteria*, phage lambda, and numerous novel viruses (Zhang et al. 2009; Coffey et al. 2014). Furthermore, novel bacteria have also been uncovered in the mosquito midgut (Lindh et al. 2005), as well as bacterial microflora in mosquito larval stages (Chavshin et al. 2012). Numerous infectious agents can be found in individual mosquitoes; 20 genera were found in more than 80% of individual mosquitoes and 60 in more than 50% (Ng et al. 2011; Boissiere et al. 2012). Overall, the mosquito midgut carries almost all the infectious agents associated with cancer, by both IARC and other researchers.

In this framework, it seems conceivable to hypothesize that a variable number of infectious agents can inhabit a niche in different parts of the body, playing a synergistic role in carcinogenesis. An IARC monograph (100B 2012) links the following: human papilloma virus with cancers in the uterine cervix, *H. pylori* with stomach cancers, hepatitis B and C with liver cancers, *C. sinensis* and *O. viverrini* with cancers of the biliary tract, and *Schistosoma haematobium* with cancers of the urinary bladder. It is not at all clear why some of these infectious agents settle in specific locations, like the biliary tract. Possibly they are taking advantage of a pre-existing weakness at that site, or that site may have some attractive features for a specific infectious agent.

The combination of specific infectious agents transmitted by a particular mosquito may dictate what type of cancer or cancers develop at a later stage. If the combination contains several of the IARC group 1 and group 2 carcinogens, then numerous cancers may develop at different sites and at different time intervals. Quite possibly one or more of these infectious agents are a catalyst, and without it cancers will not develop. If a catalyst is a necessary component of cancer development, then it may be found only in a very small number of mosquito species. It may

be an endogenous infection peculiar to that species, or it may be acquired exogenously from intermediate hosts not utilized by other species. For example, some mosquito species have very specific hosts, and a rare *Plasmodium* parasite has been recorded only in skylarks (Zehtindjiev et al. 2012).

14.5 Current Beliefs with Respect to Cancer Causation

While what we are suggesting here dovetails nicely with the known relationship of certain infectious agents to cancer, how does it relate, if at all, to other beliefs, namely, that cancers are caused by various environmental, genetic, or nutritional factors? We maintain that it can. With respect to environmental factors, sewage works are known to support high insect densities (Fang 2010) and may contain residues of medicinal and recreational drugs. Bats provide evidence that abiotic substances can be picked up by insects. Bats, with their diet of insects, have been found to contain organochlorine pesticides, polybrominated diphenal ethers, salicylic acid, and thiabendazole (Fang 2010; Secord et al. 2015). Mosquitoes are known to breed in water puddles and flooded areas containing biotic and abiotic components (Secord et al. 2015). Rainwater puddles, within a chemical plant, may absorb leaked chemical residues, and puddles present in agricultural fields are likely to contain herbicides and insecticides. Puddles developing in fields occupied by cattle, sheep, pigs, and chickens will contain their waste products, and hence diseases, as well as possibly including antibiotic residues. When mosquitoes drink from these sources, which they do, any of these contaminants may be absorbed into the midgut and subsequently injected into the human blood stream during a blood meal. Later, they may be found in any analysis of tumors. Furthermore, chemicals or other contaminants, picked up by humans during their occupation or lifestyle, may not be cancerous in themselves but may become so after being ingested by a mosquito and then mixed with other infectious agents in the mosquito midgut before being passed on to the next host.

Experiments have been undertaken to create chemical carcinogenesis in animals; the process is strongly dose-dependent and involves two stages: initiation and promotion (Miller 1978; Kato et al. 1998). With increased dosage, the animal immune system is overpowered, thus allowing the chemical agent to pursue a carcinogenic path. With present health and safety rules applied in most countries, it is unlikely that humans will be exposed to frequent or occasional high doses of chemicals. That said, cancers have been linked with chemical contamination for the same reason we propose a mosquito cause, namely, an effect on the immune system, coupled with one or more carcinogenic agents. It is probable but by no means certain that these two events need to occur contemporaneously for cancer to develop. With respect to a mosquito bite, one bite may be sufficient (acting as both initiator and promoter), or repeated (promoter) bites may be required. Ask anyone prone to mosquito bites, and they will tell you that they are constantly being bitten. We maintain that the chances of infection and the effects on the immune system

from the saliva involved with a mosquito bite, in comparison to infection from a chemical contamination, are much higher.

The IARC research group, Section of Nutrition and Metabolism, in their introduction states that low-quality nutrition, obesity, and scarce physical activity are thought to be important contributors to increasing cancer incidence; however, the mechanisms of action of these factors remain poorly understood. On October 3rd 2017, the US Centers for Disease Control and Prevention reported on the increased association between obesity and certain cancers, with obese women being more at risk than men. What they reported was an association rather than a cause, so perhaps the association is that obese humans, who often have a slight increase in body temperature compared to non obese people, are more easily targeted by mosquitoes because of the latter's ability to sense body heat (Savastano et al. 2009; Fernandez-Grandon et al. 2015). Let us not forget that animals also develop cancers (McAloose and Newton 2009; Munson and Moresco 2007), and yet they do not drink alcohol, smoke tobacco, and generally, pets apart, have sufficient nutrition and physical activity. Indeed, cancers have been found in dinosaurs (Rothschild et al. 2003).

A good deal of current medical research is being directed toward understanding the role of genetics in cancer. However, do faulty genes exist in the absence of a mosquito bite, or do they develop because of one? Even if a mosquito bite does not change the human genetic makeup, then maybe pre-existing genetic factors make some people more susceptible to mosquito bites, rather than directly leading to cancer. Female mosquitoes choose some individuals over others based on differences in volatile chemicals produced by the human body (Fernandez-Grandon et al. 2015). Where there is a family history of cancer, possibly this is partially due to them residing adjacent to a mosquito breeding or drinking location. Farmers, farm workers, and outdoor construction workers, occupations that have a higher incidence of certain cancers (see Hutchings and Rushton 2012), work adjacent to ditches, water troughs for animals, and trees in which mosquitoes are known to circulate. Furthermore, mosquitoes generally are not too choosy about which mammal they select for a blood meal. In addition to humans, they bite birds, pigs, goats, and cattle as well as pets, all of which, by their very nature, are likely to be outdoors and near farm and building workers (Tan et al. 2008).

Mosquitoes taking blood from birds, animals, and reptiles may transmit to a host a variety of *Plasmodium* parasites, as there are known to be many types, including 50 avian ones (Pick et al. 2011; Njabo et al. 2009). The suspicion that mosquitoes can cause cancers is enhanced when looking at animals such as dogs, cats, and other wild animals that contract cancers (Munson and Moresco 2007; McAloose and Newton 2009). The IARC monograph (100B 2012) shows that the Epstein-Barr virus can be transferred to primates and marmosets, and the human T-cell lymphotropic virus can be transferred to dogs and cats. The IARC states that Epstein-Barr virus is carried by mosquitoes and the human T-cell lymphotropic virus by insects (Monograph 100B 2012). Further evidence for the role of insects in animal cancers can be found in horse sarcoids. Horse sarcoids, regarded as a form of cancer by Liverpool University in the UK, are suspected of being caused by the bovine papillomavirus, and flies are suspected of being involved in the transmission of the viral

particles (www.nadis.org.uk). IARC also states that animals and humans can have the same viruses. That being so (or in the case of bovine papillomavirus perhaps slightly different strains), then they may obtain them from the same source, namely, mosquitoes.

There are other routes making a connection between mosquitoes and cancer. Artemisinin is an antimalarial product, which has been shown to cause apoptosis in several cancer cell lines, and in addition is used for treating malaria parasites, herpes viruses including EBV, hepatitis B and C, *Schistosoma*, and *C. sinensis* (Efferth et al. 2008; Li et al. 2001; Mercer et al. 2011; Yang et al. 2014; Hou et al. 2008; Singh and Lai 2004; Lai et al. 2012).

Three case studies link *Balantidium coli* infection with a leukemic patient, a patient with non-Hodgkin lymphoma, and a patient with anal cancer, respectively (Anargyrou et al. 2003; Yazar et al. 2004; Vasilakopoulou et al. 2003). *Balantidium* is an opportunistic parasitic pathogen with a worldwide presence in pigs and pig fecal matter in water (Schuster and Ramirez-Avila 2008), from which mosquitoes may drink.

Different mosquito biotypes carry different microorganisms, for example, the British *Culex pipiens* carries a *Wolbachia* strain that is not found elsewhere in Europe (Malcolm 2009), and various strains of Herpes viruses could explain why different types of cancer are more prevalent in some countries or continents than others (IARC monograph 100B 2012).

IARC states that only 20% of cancers in total have been associated with infectious agents (Monograph 100B 2012), which would suggest that mosquitoes play only a bit part in cancer development, but is that figure understated and if so why? For instance, the identified causes of encephalitis only amounted to 2575 out of 6414, and the authors were concerned about data sources (suggesting underreporting) and concerned about the absence of specific diagnoses (Davison et al. 2013). Furthermore, in another study, 37% of samples had unknown etiology (Granerod et al. 2010). Therefore, infectious agents linked with cancer may be similarly underreported, due to the cost of searching for the variety of infectious agents that could be involved and the factors raised in section F. Research on co-infections exceeding three infectious agents is unavailable, despite the number of infectious agents found in cancers by all researchers and those in the mosquito midgut (Ward et al. 2016). The IARC connects 20% of cancers to 9 infectious agents; however, if most of the 33 infectious agents are involved in some way, as suggested here, then 20% is likely to be well short of the real percentage. The fact that mosquitoes have also been shown to carry unidentified viruses and bacteria suggests that the 33 infectious agents presently suggested as having a possible role in cancer development may also be significantly below the reality. Besides, the polyomaviruses, particularly SV40, have not been proven to exist in mosquitoes so far. It is theoretically possible for mosquitoes to transmit polyomaviruses as this family of viruses exists in avian hosts, favored by some mosquito species (Feltcamp et al. 2013). In this framework, Ward and Benelli (2017b) recently suggested that the mosquito *Culiseta annulata* (Schrank) may transmit four out of the nine IARC group 1 carcinogenic infectious agents (i.e., hepatitis C, human papilloma virus, HTLV type 1, and *C. sinensi*) and six other infectious agents linked with cancer.

14.6 Can the Human Immune System Cope with Infectious Agents Transmitted by Mosquitoes?

The IARC states the importance of immunosuppression in the development of cancer and raises the immunosuppressive role of transplants and HIV: "whose main mechanism of cancer induction is impairment of the immune system and, hence, enhancement of the probability of a wide spectrum of infections including cancer-associated ones" (IARC Monograph 100B 2012).

Immunosuppression is defined by the Miller-Keane dictionary as inhibition of the immune response to unfamiliar antigens. The Farlex Partner medical dictionary defines immunosuppression as prevention or interference with the development of immunologic unresponsiveness (tolerance); it may be artificially induced or may be caused by disease. The IARC's emphasis is on induced immunosuppression.

However, while it recognizes the immunosuppressive role of mosquitoes in Burkitt's lymphoma, we believe more emphasis could be placed on the effects on the immune system of mosquito saliva both locally and more widely (Depinay et al. 2006; Schneider and Higgs 2008). Mosquito saliva, from different species carrying different viruses, leads to "an increase in virus transmission, host susceptibility, viremia, disease progression and mortality" (Schneider and Higgs 2008).

Furthermore, as well as the effects on the immune system of mosquito saliva, it has also been shown that transmission could be a further factor as "mosquito bites down-regulate the Ag-specific DTH response by a mechanism dependent on mast cells and mediated by IL-10" (Khazare et al. 2011). Mast cells are commonly seen in tumors, and their role in the control of innate and adaptive host immunity can influence responses to cancer, with suggestions that they may increase tumor development (Khazare et al. 2011). As a consequence of a mosquito bite, the immune system is affected by both saliva and a variety of infectious agents including carcinogenic ones (Benelli et al. 2016a).

We have seen so far that mosquitoes can transmit both numerous infectious agents (Table 14.1) and saliva, which raises another important question. Can the immune system recover from such an onslaught, and can it protect us from any later possible development of cancer? The answer is much more complex than might be imagined. The immune system and infectious agents are constantly engaged in a war for supremacy. As in any war, new technologies and tactics are discovered and employed for one side to gain an advantage. The literature shows how this war between the immune system and disease is being played out. The strategies employed by infectious agents associated with cancer are fascinating, numerous, and complex, and there are some excellent and comprehensive reviews (see Sen 2001; Finlay and McFadden 2006; Schmid-Hempel 2009). Firstly, however, we provide a brief description of how the immune system operates.

The immune system has both innate and adaptive responses; the innate immediately defends the host by means of neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins. The adaptive response is less immediate and has memory (Parkin and Cohen 2001). Pathogen recognition receptors (PRRs)

Table 14.1 Infectious agents with immune evasion strategies and links with both cancer and mosquitoes

Infectious agent with evasion capacity	Linked with cancer	Linked with mosquitoes	References
Adenovirus	Yes	Yes	Sevik (2012)
Cytomegalovirus	Yes	Yes	Ng et al. (2001)
Epstein-Barr virus	Yes	Yes	Ng et al. (2001)
Hepatitis B virus	Yes	Yes	Fouche et al. (1990)
Hepatitis C virus	Yes	Yes	Chang et al. (2001)
Human papilloma virus	Yes	Yes	Ng et al. (2001)
Kaposi's sarcoma virus	Yes	Yes	Ng et al. (2001)
Helicobacter pylori	Yes	Yes	Gupta et al. (2012)
Mycobacterium tuberculosis	Yes	Yes	Chavshin et al. (2012)
Salmonella enterica	Yes	Yes	Boissiere et al. (2012)
Staphylococcus aureus	Yes	Yes	Boissiere et al. (2012); Minard et al. (2013)
Streptococcus pyogenes	Yes	Yes	Boissiere et al. (2012); Minard et al. (2013)
Helminths	Yes	Yes	Brooker et al. (2007)
Plasmodium falciparum	Yes	Yes	Carcinogenic agent falling into the IARC group 2A

are used by the immune system to sense invading pathogens. These PRRs identify specific pathogen-associated molecular patterns (PAMPs) produced during infection (Munoz-Jordan and Fredericksen 2010).

The immune system responds to infectious agents by deploying interferons and specialized cells such as phagocytes. These specialized cells can internalize and destroy microbes and in addition recruit more immune cells (Finlay and McFadden 2006). Interferons (IFNs) are proteins (called cytokines) that inhibit virus replication and have pleiotropic effects on many aspects of cell physiology, including cell growth, cell motility, and cell functions. Interferons consist of type I and type II; each acts through different cell surface receptors and is structurally unrelated (Sen 2001). There are many members of the type I superfamily but only one member of the type II family. An IFN-induced protein PKR13 has a pivotal role in host-virus interactions manifested by the fact that numerous viruses block its activation or action using a variety of biochemical strategies (Sen 2001). The ways in which pathogens evade or modulate the immune response are both numerous and complex.

Finlay and McFadden (2006) set out numerous strategies that viruses and bacteria use to avoid immune recognition, response, and destruction, while Schmid-Hempel (2009) reveals parasite strategies and factors surrounding pathogenesis and virulence.

Here, we will examine how the immune system responds to some of the infectious agents known to be carcinogenic. We separate them into firstly the IARC

group 1 carcinogens and secondly into the remainder (which we do not claim as carrying the same weight). The herpes viruses (which includes EBV) can secrete viral modulators, subvert phagocytes, and cause dysfunction of the NK cells that are part of the development of acquired cellular immune responses, and CMV in particular (being considered for group 1 status) can induce the expression of cellular complement inhibitors (Finlay and McFadden 2006). Sen (2001) describes how EBV and CMV block different parts of the IFN system. The herpes family of viruses also have latent viral tissue culture systems, and during latency, the viral genome replicates in tandem with the host cell using the latter's replication machinery (Moore and Chang 2010). HHV-6A, a member of the herpes family, has for the first time been implicated in female unexplained infertility development and possibly able to infect cervical cells (Marci et al. 2016). HHV-6A either in latent form or during acute infection can also activate the human endogenous retrovirus K-18 that can cause deregulation of the immune system (Tai et al. 2009). PCR analysis of peripheral blood mononuclear cells and saliva failed to find HHV-6A DNA, illustrating that blood and saliva tests may produce false negatives (Higashimoto et al. 2012).

Hepatitis C is quoted as having antigenic drift that can evade the immune response (and obstruct vaccine production) and manipulate signalling by Toll-like receptors (Finlay and McFadden 2006). Hepatitis C virus evades antiviral systems by modulating the function of innate immune proteins, including the signalling adaptor protein MAVS (mitochondrial antiviral-signaling protein) (Gokhale et al. 2014). Research shows how PAMPS trigger signalling and that HCV, EBV, KSHV, and *Papillomaviridae* have techniques to counteract the immune responses (Navratil et al. 2010).

HTLV 1 can inhibit complement by incorporating host inhibitors into the virus envelope (Finlay and McFadden 2006). *H. pylori* can interfere directly with acquired immunity by blocking type 1T helper cell development (Finlay and McFadden 2006). *Schistosoma* spp. are said to produce competing ligands to impede recognition by the host (Schmid-Hempel 2009). Human papilloma virus can block two out of the four parts of the IFN system (Sen 2001).

We also have to address myths, for example, prostate cancer is one of the most common cancers in men, and the generally accepted view is that it needs the hormone testosterone to grow (Cancer Research UK 2017). This latter contention is disputed on the basis that testosterone and its relation to prostate cancer are based on just one patient's blood test result obtained in 1941 (Morgentaler 2006). Two common viruses, human papilloma virus (HPV) and Epstein-Barr virus (EBV), both linked with mosquitoes are present and may be collaborating with each other in much of prostate cancer cases (Whitaker et al. 2012). Both viruses were found in more than half of the malignant cases, and HPV alone was present in about 70% of the malignant prostate cancers sampled. Zur Hausen (1996) also found that HPV was responsible for cancers of the cervix, and HPV has subsequently been linked to 70% of those cancers.

Other infectious agents that have been linked with cancers and which possess immune evasion strategies include *Salmonella*, which has developed means of

altering molecules on their surface to make them less identifiable to the immune system. They also have means to neutralize phagocytic activity and generally suppress apoptotic death (Finlay and McFadden 2006). *Salmonella* also prevents the delivery of (toxic) oxidase into its self-made vacuole (Schmid-Hempel 2009). *Streptococcus pneumoniae* relies extensively on its capsule to prevent antibody and complement deposition on its surface, thus avoiding phagocytic clearance, *Streptococcus pyogenes* have effectors that can alter immune functions to enhance immune evasion, and various streptococci can secrete enzymes such as IgA proteases that degrade immunoglobulins (Finlay and McFadden 2006). *S. aureus* targets cytokine pathways to enhance pathogenesis and can produce toxins that lead to a generalized immunosuppression (Finlay and McFadden 2006; Janeway et al. 2001). *M. tuberculosis* has numerous surface glycolipids and carbohydrates that prevent phagosome acidification and alter phagosomes (Finlay and Mcfadden 2006; Janeway et al. 2001).

A principal mosquito parasite, *P. falciparum* (and presumably other species), can store approximately 60 surface variants that are successively expressed in order to evade immune recognition and have been reported to produce competing ligands to impede host recognition (Schmid-Hempel 2009). WHO (2016) states that vector-borne diseases account for more than 17% of all infectious diseases, causing more than 1 million deaths annually, and that more than 2.5 billion people in over 100 countries are at risk of contracting the mosquito-transmitted dengue fever. Known mosquito-related infections feature strongly in the immune-evasion literature, for example, malaria has mechanisms for evading cytotoxic T cells and controlling protein export during liver infection to minimize immune recognition (Bertolino and Bowen 2015; Corradin and Levitskaya 2014; Montagna et al. 2014). It is also proposed that severe disease linked with dengue virus and WNV infections correlates with their ability to counteract the IFN-α/β response (Munoz-Jordan and Fredericksen 2010). Also, Japanese encephalitis virus has mechanisms for coping with immune responses (Lin et al. 2004).

Blázquez et al. (2014) reviewed the stress responses in flavivirus-infected cells. The flaviviruses include dengue virus, yellow fever, WNV, Japanese encephalitis virus, and Usutu and Zika viruses. These viruses can trigger one or more of the three arms of the unfolded protein response (UPR). The neurovirulent WNV NY-99 upregulates all three pathways of the UPR. Interaction between the UPR and interferon signaling in flaviviral infections has been reported, and there is evidence of connections with cellular metabolism, apoptosis, and innate immunity, although the mechanistic links are unclear. WNV infections are reportedly more virulent when there is impaired immune protection (James et al. 2016). CMV infection results in significantly weaker responses to superinfection with influenza, human herpes virus, or WNV (Cicin-Sain et al. 2012). Polyomaviruses, which may be present subclinically in many people, may become more problematic infections in patients whose immune system is impaired (Allander et al. 2007; Babakir-Mina et al. 2011; Abedi Kiasari et al. 2011; Dalianis and Hirsch 2013).

IARC and recent research agreed cancer viruses do not belong to a single viral class; they cover the entire range of virology and include retroviruses, positive-

stranded RNA viruses, and both large and small double-stranded DNA viruses (Moore and Chang 2010). The authors identify immunity as an important ingredient in whether pursuant to acquiring a potential cancer virus cancer actually develops. The evidence they provide is that Kaposi's sarcoma herpesvirus (KSHV) caused less than three Kaposi's sarcoma cases per year in the USA, but the rate increased tens of thousands-fold in patients suffering AIDS because of immunosuppression arising from HIV. As viral and bacterial diseases are frequently linked, they can take advantage of weaknesses created by another pathogen (Finlay and McFadden 2006).

In some examples, virulence emerges because the infection enters a part of the body that, on the face of it, has nothing to do with transmission. Bacteria on occasions may need to extract host resources for its survival, for example, extracting iron (Damian 1997), and some parasites can feed on the host's immune response; for example, *Leishmania* grows by consuming cytokines, and tapeworms use antibodies as nutrients (West 2002).

In both birds and humans, there is individual variation in pathogen susceptibility due to underlying variations at different parts of the immune system; individuals are affected differently once they become infected, and innate genes are constantly interacting and evolving with a changing pathogen flora (Hellgren 2015).

Relevant to cancer is also the concept of "hit-and-run" viruses in which the viral genes are lost as the tumor begins to mature (Moore and Chang 2010; Shen et al. 1997). Johansson and Ward (2016) raise a bake-a-cake hypothesis in which the infectious agents and saliva are mixed and then slowly expand into a tumor, in the same way that a cake is baked.

Parasites are able to successfully infect a wide range of host species of broad ancestry and able to be the most prevalent in single host species (Hellgren et al. 2009; Webster et al. 2016). Furthermore, avian malaria parasites have developed evolutionary independence (Hellgren et al. 2015). The literature shows that a single person can be bitten by a mosquito a thousand times per annum (Ferguson et al. 2010), so imagine how many infectious agents that person may pick up, together with continuous effects on the immune system arising from mosquito saliva. It is known that tumors contain numerous infectious agents; the question is how did they get there, and what is their relationship to cancer development? The present assumption is that the immunosuppressed, brought about through HIV, after transplants, or developing cancer, are then prone to catching infectious agents in a linear fashion.

However, is this assumption realistic? In most developed countries, water, food, and hygiene standards are such that acquiring the number of infectious agents shown to be present in tumors should be minimal, whereas acquiring them from a mosquito bite seems much more likely. Various studies suggest that the effective dose for successful infection may often be surprisingly small and that co-operatively acting infectious agents tend to have higher infective doses than infectious agents where the factors are injected individually (Schmid-Hempel 2009).

In summary will the immune system respond to a mosquito bite by regaining control? If the mosquito transmits several cancer-related infectious agents, the answer is likely to be no; on the other hand, if some mosquito species cannot transmit cancerous agents and/or some people are better adapted than others to

deal with particular infections, the answer may be yes (Hellgren 2015). Other exogenous factors may influence developments, for example, it has been speculated that disturbance of the immune system by electromagnetic fields is a potentially underlying cause for cellular damage and tissue repair reduction (Johansson 2009). As it is now known that male mosquitoes can carry similar infectious agents to the female (Anderson et al. 2006) and that pathogens have been discovered in mosquitoes from wild-caught larvae (Chavshin et al. 2012), we should assume that mosquitoes are endogenous carriers of various overlooked infectious agents. If the endogenous infections are both uniform and potentially cancer-causing, then reliance on intermediate hosts may be less important than supposed.

14.7 How Has the Mosquito Escaped Identification as a Suspect in Cancer Development?

Zoonoses are recognized as being responsible for 60% of emerging infectious diseases (Jones et al. 2008), and yet so far, with respect to cancer, the mosquito has only been implicated in Burkett's lymphoma (Banfield et al. 1966), and *Plasmodium* has been given a 2a IARC rating rather than group 1. This maybe because of the wide diversity of *Plasmodium* species. Simian *Plasmodium knowlesi* has been shown to infect humans while commonly mis-diagnosed as benign *P. malariae* (Cox-Singh et al. 2008). Unknown lineages of simian *Plasmodium* have recently been found to which humans may be susceptible (Krief et al. 2010). This raises the intriguing possibility that other varieties of malaria, for example, avian ones, may, contrary to previously accepted opinion, be capable of survival in humans (Ward and Benelli 2017a) and participate in some way in the development of cancers. In fact, generally accepted opinion may be a myth, arising from unreliable tests in the past, failing to detect either simian or avian malaria in humans, or that low levels of parasitemia have been found and deemed to have no known adverse health consequences (Ward and Benelli 2017a).

If it can be shown that different varieties of avian malaria can remain viable or even dormant in humans, it would reinforce the argument that cancers may be more intertwined with mosquitoes than imagined. Avian malaria parasites are present throughout the world except for Antarctica (Braga et al. 2011). These other "nonhuman" malarias may be avirulent strains behaving as innocent bystanders or participating in a way not yet understood. Mosquito-vectored viruses have a presence in healthy UK birds but are either avirulent or the birds have built up high levels of immunity, as they appear to have no outward clinical signs of infection (Buckley et al. 2003).

If such malarias exist, but remain asymptomatic in humans, should we be concerned or interested? The answer should be yes, as finding them would enable an assessment to be made of the percentage of total cancers attributable to mosquitoes and also offer the prospect of a single universal cancer test. Furthermore, if an avian or simian malaria is actively implicated in cancer development, then blood donors would need to be screened for it (Allain et al. 2009; Dodd 2010).

We already know that mosquito species carrying, or capable of being bridge vectors for both simian and avian malaria, cannot only bite humans but give them serious diseases, for example, *Culex* mosquitoes infected with *Plasmodium relictum* have killed birds, horses, and humans through transmission of WNV (Spielman and D'Antonio 2001; Buckley et al. 2003; Hughes et al. 2010; Peterson et al. 2013). WNV-related illnesses are often unrecognized clinically with one study showing only 58% of patients had a positive MAC-ELISA result at clinical presentation (Peterson et al. 2013). Also, different *Plasmodium* species can vary asynchronously throughout the season in both mosquitoes and birds (Medeiros et al. 2016).

14.8 Apart from *Plasmodium*, What About Our Detection Rate of Other Known Carcinogens in Cancer Patients?

The UK National Health Service (NHS) has a range of information sheets on viruses linked with cancers. With respect to the herpes viruses, the information sheet for EBV states that 95% of people in the UK are thought (but not proven) to be infected by the time they are 40 years old, so it is unlikely that cancer patients will be routinely checked for EBV. Similarly, the information sheet for CMV states that it is mainly asymptomatic, and it is thought that 50–80% of adults in the UK are infected. HHV-8 will be looked for in potential cancer patients should they have the characteristic discolored patches.

Hepatitis B and C will be discussed but not routinely checked for when a patient is migrating or returning from a trip to a country where these viruses are known to be present. Skin stretch tests may be used, even though there is no reported validation of stiffness cutoffs and noninvasive tests are poor for fibrosis (Degos et al. 2010; Tsochatzis et al. 2011). HCV is often asymptomatic until extensive liver damage. When suspected, a blood test is carried out; however, diagnosis of HBV-positive hepatocellular carcinoma (HCC) is problematic independent of cirrhosis etiology, because of a lack of biomarkers (Li et al. 2010). There are no reliable figures for HBV and HCV involvement in worldwide disease (Perz et al. 2006). Human T-cell lymphotropic virus (HTLV) is only referred to in factsheets by way of non-Hodgkin lymphoma. This can be observed through lymph node swelling, but occasionally non-Hodgkin lymphoma first develops in an organ or outside the lymphatic system. It is worth noting that risk factors include a *H. pylori* infection or EBV infection (both recognized as IARC group 1 carcinogens). However, as EBV infection is regarded as widespread and *H. pylori* fairly common, there is little likelihood that finding either of these would instigate HTLV tests.

Genital HPV, according to factsheets, is a component of the cervical screening that is offered to women between the ages of 25 and 64. There isn't a reliable test for males, where HPV is known to give rise to over 70% of prostate cancers, as different types of test can produce different outcomes (Ronco et al. 2013). Although HPV has been linked with mosquitoes (Ng et al. 2011), this connection is not routinely checked for in cervical and prostate cancers.

Lastly, *H. pylori* as such does not appear in NHS factsheets, but *Campylobacter* (a food poisoning agent) does. It makes no mention of a cancer connection, and there is no suggestion it would be looked for in cancer patients.

Schistosomiasis is caused by a parasitic worm that lives in fresh water in subtropical and tropical regions (see Melhorn 2016 for a recent review). Infected patients are frequently asymptomatic at the outset although organ damage can occur later (Figueirido et al. 2015; Machicado and Marcos 2016). Diagnosis usually requires an expert in tropical diseases, so cancer patients residing outside of the tropics and subtropics would not be routinely tested for this, despite increased levels of travel and migration (Akpata et al. 2015).

C. sinensis and *O. viverrini* are not mentioned in any NHS factsheets, so it would be reasonable to suppose they would not be routinely tested for in cancer patients. In any event the "gold standard" detection method for *O. viverrini* has limited diagnostic sensitivity and diagnostic specificity (Worasith et al. 2015).

The NHS sets out numerous tests for a multitude of different cancers and lists a large set of symptoms; however, a single test is not yet available, and as described above, there are no routine tests for group 1 carcinogens.

14.9 Casting a Critical Eye on Cancer Detection and Mosquito Control

Until a significant mosquito-cancer connection is proven and new more reliable tests for identifying cancer are discovered, there will be little or no change to the current practices. Because some carcinogenic infectious agents can overpower the human immune system, developing vaccines and controlling these agents, even if they are detected, is extremely difficult (Finlay and McFadden 2006). Viruses in tumors are generally latent; thus, antiviral drugs targeting the viral replication machinery are ineffective in treating mature tumors (Moore and Chang 2010). It has been reported that the most advanced tetravalent live-attenuated dengue fever vaccine candidates showed a poor overall efficacy rate (30%) in a recently published phase 2 clinical trial (Zhang et al. 2013).

Very few people infected with infectious agents linked with cancer go on to develop tumors. However, cancers that are linked to immunosuppression are suspects for being caused by tumor viruses as infective dosages can be small. As stated earlier mosquito saliva can affect the immune system, and mosquitoes can carry a multiplicity of infectious agents, some known to be carcinogenic and in themselves having different strategies to evade the immune system (Johansson and Ward 2016). Whether the immune system can regain equilibrium may well depend on how many carcinogenic infectious agents are transmitted, either from one or multiple bites and individual immune system characteristics.

Besides the overlooked issues on cancer development and detection mentioned above, the development of effective and sustainable control tools to manage mosquito populations is still of timely importance (Benelli and Beier 2017). There are two broad approaches to mosquito control, namely, personal and public actions, and

they are not mutually exclusive. Individuals must focus on bite avoidance, using insect repellents or physical controls. It is doubtful that all individuals will be able or willing to take steps to avoid being bitten, as this may involve continuous use of repellent, removal of garden water features, and maybe uprooting trees, bushes, and flowers known to harbor or provide food for mosquitoes.

Individuals could reduce the likelihood of being bitten by putting larvivorous fish into garden ponds, removing other water sources from close-related domestic habitats, wearing more appropriate clothing when outdoors (including insecticide treated ones), and using personal repellents (Mehlhorn 2012; Lupi et al. 2013), particularly at times when mosquitoes tend to be more active. Moreover, they can protect biodiversity in their gardens avoiding the use of broad-spectrum insecticides and supporting the species that consume mosquitoes as part of their diet (see Benelli et al. 2016b for a review). The use of green synthesized nanoparticles as novel pesticides has interesting potential (Benelli 2016a, b) but requires further investigation regarding their environmental impact (Benelli et al. 2017a, b, c).

Public control programs targeting mosquitoes involve national and local authorities stepping in to deal with major outbreaks of mosquitoes. For instance, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) contributed substantially to the reduction of malaria cases in sub-Saharan Africa (Benelli and Beier 2017). More recently, novel control tools, such as "eave tubes" (Sternberg et al. 2016; Waite et al. 2016) and attractive toxic sugar baits (ATSB) (Beier et al. 2012), are obtaining highly promising results. Also, the sterile insect technique (SIT) is a candidate as a novel and eco-friendly control tool; briefly, it is a self- limiting population suppression system, whereby radiation-sterilized male insects are released to mate with their wild counterparts, thereby reducing the reproductive potential of the targeted population (Bouyer and Lefrançois 2014; Bourtzis et al. 2016). Moreover, there is considerable excitement at the prospect of releasing mosquitoes infected by *Wolbachia* (maternally inherited bacteria inducing cytoplasmic incompatibility in these insects, see Benelli et al. 2016b for a review), as well as genetically modified male mosquitoes in the hope that they will, if released in large quantities, outcompete their wild counterparts and mate with, but not breed with, available females (Bourtzis et al. 2016).

Besides, local authorities should deal with the possible causes of mosquito proliferation, for example, filling in newly created water sources in the environment, arising perhaps from extractive industry waste sites, and dealing with the mosquitoes themselves. This is not an easy task, as illustrated by several comprehensive reviews of the biological complexities involved in environmental mosquito control (Ferguson et al. 2010; Benelli and Mehlhorn 2016; Webster et al. 2016). The authors conclude that mosquitoes need to be controlled at multiple points in the continuum from egg to adult, especially in urban areas, while – on the other hand – we should keep in mind that targeting young instars is not advisable in rural environments, as clearly outlined by integrated vector management criteria (Benelli and Beier 2017). Overall, in agreement with IVM, we place emphasis on mosquito management rather than eradication, since allowing a pest population to survive at a reasonable threshold reduces selection pressure and the growth of novel, more resistant, and aggressive populations.

14.10 Conclusions and Future Challenges

The risk of developing cancer is increased by deliberate immunosuppression, but what about the effects that mosquito saliva has on the immune system? As mosquitoes are ubiquitous, their saliva, coupled with infectious agents, which it is known to transmit, will have effects on the immune system. Zoonoses contribute to a high percentage of human diseases, and mosquitoes transmit disease from animal, bird, and reptile to humans. Individual mosquitoes are known to carry numerous infectious agents, including *Borrelia* (responsible for Lyme disease), and we have observed that persons prone to bites are frequently bitten. Further research is required to determine what level of contribution mosquitoes may play in the increasing incidence of Lyme disease. Being bitten could be the equivalent of being injected by a drug user's dirty needle left in the street. However, as cancer takes time to develop, the mosquito has so far evaded suspicion, whereas with other common mosquito-borne diseases, symptoms and sometimes death occur fairly quickly.

There is a significant body of literature that provides circumstantial evidence, but as yet no proof, of a mosquito-cancer connection (see also Benelli et al. 2016a, b). Many infectious agents linked with cancer can be found in mosquitoes; furthermore, some of the infectious agents, particular those of the herpes family, have evolved means of avoiding immune system control. Additionally there does not appear to be a systematic testing for infectious agents in cancer patients, and this may have allowed the mosquito to slip under the radar. Controlling mosquitoes is a top priority in any event, and if the connection to cancer can be proven beyond all doubt, then the importance of control will be even more urgent. Different control measures exist with laudable attempts being made by scientists in the field of genetics. However, with such a dangerous opponent, are the unknowns connected with genetically altering, or in other ways tampering with mosquitoes themselves, too large a risk to take? Other intelligent solutions are or could be made available which may be just as cost-effective in the long run.

Conflict of Interest The authors declare no competing interests.

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