Antiviral responses of arthropod vectors: an update on recent advances

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Abstract Arthropod vectors, such as mosquitoes, ticks, biting midges and sand flies, transmit many viruses that can cause outbreaks of disease in humans and animals around the world. Arthropod vector species are invading new areas due to globalisation and environmental changes, and contact between exotic animal species, humans and arthropod vectors is increasing, bringing with it the regular emergence of new arboviruses. For future strategies to control arbovirus transmission, it is important to improve our understanding of virus-vector interactions. In the last decade knowledge of arthropod antiviral immunity has increased rapidly. RNAi has been proposed as the most important antiviral response in mosquitoes and it is likely to be the most important antiviral response in all arthropods. However, other newly-discovered antiviral strategies such as melanisation and the link between RNAi and the JAK/STAT pathway via the cytokine Vago have been characterised in the last few years. This review aims to summarise the most important and most recent advances made in arthropod antiviral immunity.

Keywords Arbovirus · Innate immunity · RNAi · Mosquito · Tick · Midge

Introduction

Arthropod-borne viruses (arboviruses) pose an increasing threat to both human and animal health worldwide, in particular in developing countries. Due to environmental change and globalisation, arboviral vectors such as mosquitoes, ticks, sand flies and (biting) midges are invading new geographic regions [17]. Most arboviruses belong to the RNA virus families Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae and Togaviridae (Table 1). Viruses with RNA genomes have a high mutation rate [31] and can be associated with a broad host range and high zoonotic potential. It is not surprising that highly pathogenic arboviruses such as chikungunya virus (CHIKV) [19, 95], severe fever with thrombocytopenia syndrome virus (SFTSV) [132], Heartland virus [79, 100] and possibly a new dengue virus (DENV) serotype [90], among others, have emerged or re-emerged over the past decade. CHIKV shows the impact of adaptation to arthropod vectors on arbovirus spread. In the 2005–2006 outbreak of chikungunya fever on the Indian Ocean island of La Reunion, rapid replication and dissemination of this infection was associated with a single point mutation in the viral envelope glycoprotein which adapted the virus to replicate in and to be disseminated by anthropophilic *Aedes albopictus* mosquitoes [121]. All arboviruses have to deal with at least two very different immune systems and, while research into and knowledge of arbovirus-host interaction in vertebrate systems is well developed, knowledge of vector antiviral responses has been gained principally over the last 10 years [32, 34, 62, 81]. This review aims to summarise knowledge to date about antiviral responses of arthropod vectors to arboviruses.

Recent advances in high throughput technologies, such as transcriptomics and proteomics, for pathogen-host

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Table 1 Arbovirus families, representatives of medical, veterinary or scientific importance and their primary vectors

Genome	Family	Genus	Important viruses	Primary vector	Ref.
ss(+) RNA	Flaviviridae	<i>Flavivirus</i>	Dengue virus	<i>A. aegypti</i>	[40]
			West Nile virus	<i>Culex</i> spp	[40]
			Yellow fever virus	<i>Aedes</i> spp	[40]
			Japanese encephalitis virus	<i>Aedes</i> spp	[40]
			St Louis encephalitis virus	<i>Culex</i> spp	[40]
			Tick-borne encephalitis virus	<i>Ixodes</i> spp	[40]
			Kyasanur Forest disease virus	<i>Haemaphysalis</i> spp	[40]
ss(+) RNA	Togaviridae	<i>Alphavirus</i>	Chikungunya virus	<i>Aedes</i> spp	[48]
			Venezuelan equine encephalitis virus	<i>Aedes</i> spp	[48]
			Semliki Forest virus	<i>Aedes</i> spp	[78]
			O'nyong nyong virus	<i>Anopheles</i> spp	[127]
ss(−) RNA	Rhabdoviridae	<i>Vesiculovirus</i>	Vesicular stomatitis virus	<i>Phlebotominae</i>	[69]
			Chandipura virus	<i>Phlebotomus</i> spp	[80]
			Bovine ephemeral fever virus	<i>Culicoides</i> spp	[86]
ss(−) RNA segmented	Bunyaviridae	<i>Orthobunyavirus</i>	La Crosse virus	<i>Aedes triseriatus</i>	[48]
			Oropouche virus	<i>Culicoides</i> spp	[15]
			Schmallenberg virus	<i>Culicoides</i> spp	[15]
		<i>Phlebovirus</i>	Rift Valley fever virus	<i>Aedes</i> spp	[48]
			Heartland virus	<i>Amblyomma</i> spp (unclear)	[79, 100]
			Severe fever with thrombocytopenia syndrome virus	<i>Haemaphysalis</i> spp (unclear)	[132]
		<i>Nairovirus</i>	Uukuniemi virus	<i>Ixodes</i> spp	[51]
			Crimean-Congo haemorrhagic fever virus	<i>Hyalomma</i> spp	[66]
			Nairobi sheep disease virus	<i>Rhipicephalus appendiculatus</i>	[66]
ss(−) RNA segmented	Orthomyxoviridae	<i>Thogotovirus</i>	Thogoto virus	<i>Rhipicephalus</i> spp	[43]
dsRNA segmented	Reoviridae	<i>Orbivirus</i>	Bluetongue virus	<i>Culicoides</i> spp	[76]
			African horse sickness virus	<i>Culicoides</i> spp	[76]
			Tribeč virus	<i>Ixodes</i> spp	[23]
dsDNA	Asfarviridae	<i>Coltivirus</i>	Colorado tick fever virus	<i>Dermacentor andersoni</i>	[7]
		<i>Asfivirus</i>	African swine fever virus	<i>Ornithodoros</i> spp	[30]

interaction studies [25, 52, 96, 109, 117, 136], as well as the publication of several vector species genomes including the mosquitoes *Culex quinquefasciatus* [2], *Anopheles gambiae* [49] and *Aedes aegypti* [89] and the tick *Ixodes scapularis* [46], have led to the discovery of immunity pathway orthologues in these species [65, 105]. While there is no midge genome available to date, recent combined efforts of The Pirbright Institute and the European Bioinformatics Institute (part of the European Molecular Biology Laboratory; EBI-EMBL) have led to the first full *de novo* sequencing project for *Culicoides sonorensis*. The *C. sonorensis* genome will provide researchers with a powerful resource for this important insect vector group, allowing

scientists to exploit new genomic technologies to elucidate the mechanisms of the *Culicoides*-vector/pathogen/host relationships (Mark Fife and Simon Carpenter, personal communication). RNA interference has been a useful tool to interrogate the function of the genes discovered through these bioinformatics approaches [29, 99, 107].

The first challenge arboviruses have to face in the arthropod is crossing the midgut barrier, which is often an important factor determining vector competence [3, 13, 83, 114]. Once the virus has successfully crossed the midgut barrier, the virus has to be able to evade the arthropod defences sufficiently to disseminate to the salivary glands and from there to the next susceptible host.

RNA interference as an antiviral defence in mosquitoes, biting midges and ticks

Among arbovirus vectors, mosquitoes are the most thoroughly-studied group with regard to their immune system. RNA interference (RNAi), identified in mosquitoes 10 years ago [47, 61], is considered to be the most important antiviral mechanism [14]. There are three major types of small RNA systems—the microRNA (miRNA), the PIWI-interacting RNA (piRNA) and the small interfering RNA (siRNA) pathways, all of which can play a role in arthropod antiviral defences (recently reviewed by [32, 122]). While the generation of exogenous siRNAs has been implicated in antiviral defences in arthropods, miRNAs and piRNAs have mainly been associated with regulation of gene expression and control of transposons in germlines (reviewed by [5, 55]); however, recently their role in virus infection has become more apparent. Numerous small RNA profiles of arbovirus-infected mosquitoes or mosquito cells have been published [45, 68, 84, 111, 123] and have provided insights into the importance of the different small RNA pathways during arbovirus infection. Knowledge gained about antiviral RNAi over the last decade has also led to new techniques for virus discovery. It was shown that nearly complete virus genomes can be assembled by sequencing of small RNAs derived from invertebrates [129]. Both siRNAs and piRNAs were found to match viral genomes.

miRNAs

Among all the classes of small RNAs, miRNAs may be considered the most important for the cell as miRNAs are important regulators of gene expression in different tissues and developmental stages [5]. In *Drosophila melanogaster* the ratio of genes expressing miRNAs to genes expressing proteins is roughly 1:100 [67]. miRNAs are ~22 nt in length and are found in most eukaryotic cells; their biogenesis in insects is reviewed elsewhere [5, 12, 75]. It is widely believed that the mechanism of biogenesis is conserved among insects, including vectors such as mosquitoes and midges. A number of miRNAs have been identified in mosquitoes of different species [42, 71, 93, 112, 118, 128] and their presence in midges is to be expected. In ticks, however, our knowledge of small RNAs is limited. So far only 49 miRNAs have been identified in the *I. scapularis* genome (miRBase [41]). In ticks of the species *Haemaphysalis longicornis* miRNAs are expressed in the salivary glands with differences in the expression pattern during blood-feeding [135]. In *Rhipicephalus (Boophilus) microplus* both conserved miRNAs and tick-specific miRNAs have been identified [10] and, as in other arthropods, the pattern of expression of the *R. microplus*

miRNA changes between developmental stages and in different organs, but whether or not the pathway components are entirely conserved remains unknown.

For the vector species in which miRNAs have been identified, we know that they play an important role in development and regulation of gene expression in the uninfected organism [10, 93, 135]; whether they are involved in antiviral responses against arboviruses remains unknown. In *D. melanogaster* a number of miRNAs have been implicated in regulating immune responses (reviewed by [5, 122]), such as miR-8 [21] and the let-7 miRNA [35] which are involved in regulation of antimicrobial peptides. Recently, miRNAs involved in controlling immune responses such as melanisation have also been predicted by a computational approach in the mosquito species *A. gambiae* [118], the vector of O'nyong-nyong virus (ONNV) [127]. In *A. aegypti* mosquitoes, miRNAs are responsible for gene expression changes after a blood-meal, and one of these (aae-miR-375) regulates immune-related genes such as *cactus* and *REL1* [54]. During infection of *A. aegypti* mosquitoes with the intracellular bacterium *Wolbachia*, the bacterium uses a host miRNA to regulate a methyltransferase, which contributes to inhibition of DENV replication [133]. These findings suggest that there may be miRNAs in mosquitoes, midges and ticks that contribute to modulating the antiviral response. A new and intriguing observation is that West Nile virus (WNV) encodes a miRNA-like molecule that can modulate host gene expression [53].

piRNAs

piRNAs are 24–32 nt in length, distinct from other small RNAs as their production is Dicer-independent [120] and do not generally form hairpins or other secondary structures during biogenesis. piRNAs interact with proteins of the P-element induced wimpy testis in *Drosophila* (PIWI) subfamily (reviewed by [110]), such as PIWI, Argonaute-3 (Ago-3) and Aubergine (Aub). The detailed mechanism of piRNA biogenesis is not entirely clear and appears to vary significantly between germline and somatic cells [44], and between *Drosophila* and mosquitoes [32]. Briefly, in the model organism *D. melanogaster* the biogenesis of piRNAs is split into two pathways—primary piRNA processing and secondary processing (reviewed by [55, 110]). In primary piRNA processing, a piRNA cluster is transcribed into a primary piRNA transcript, which is then shortened into a piRNA-intermediate having the determined 5' end of the mature piRNA. This precursor then interacts with a PIWI protein, such as Ago-3, which cleaves the 3' end to result in a mature piRNA incorporated in the piRNA-induced silencing complex (piRISC). The piRISC will target complementary RNA strands and the incorporated PIWI protein

will cleave the target RNA. The second part of the pathway, the so-called ping-pong amplification loop, is not present in all cell types. Primary piRNAs are transcribed in antisense and will interact with a complementary target sense RNA which is cleaved by the piRISC and results in a secondary sense piRNA. While primary piRNAs tend to have a uridine at position one in the 5' end, secondary piRNAs often lack this characteristic, but favour an adenine at position ten. The secondary piRNA can then give rise to a primary piRNA leading to an amplification of piRNAs thereby completing the loop [55, 110].

While PIWI-mediated RNAi has been described as a mechanism of protection against transposable elements in germline cells of some arthropods and vertebrates, it has recently also been implicated in mosquito antiviral immunity. Increased knowledge about the piRNA pathway in mosquitoes reveals many apparent differences from that in *Drosophila* (reviewed by [32]). Virus-derived piRNAs have been shown to be produced in whole *Aedes* mosquitoes upon infection with CHIKV [82] and DENV [45, 104], in *Aedes* mosquito soma upon infection with CHIKV [82] and in the cell lines U4.4, C6/36 (both *A. albopictus*) and Aag2 (*A. aegypti*) upon infection with Sindbis virus (SINV) [123]. Virus-derived piRNAs were also observed in C6/36 cells infected with the bunyavirus La Crosse virus (LACV) [123]. In another study, both U4.4 and Aag2 cells produced piRNAs after infection with the alphavirus Semliki Forest virus (SFV), and knockdown of one of the PIWI proteins, PIWI4, resulted in an increase in SFV replication, demonstrating that activity of a PIWI protein was involved in controlling arbovirus replication [102]. In addition, PIWI-mediated RNAi has been observed in Aag2, U4.4 and C6/36 cells infected with the bunyavirus Rift Valley fever virus (RVFV) [68]. All three cell lines were shown to produce viRNAs with a PIWI signature, in particular late in infection after the peak of 21 nt viRNA production.

Recent progress in the piRNA field has revealed the importance of PIWI-mediated RNAi in the antiviral response in mosquitoes. The mechanism and origin of these virus-specific piRNAs remains unclear. Hoa et al. [47] have shown that the PIWI protein Ago-3 plays a role in gene silencing mediated by dsRNA treatment in *A. gambiae* cells and Morazzani et al. [82] suggested that dsRNA is responsible for the piRNA-like molecules induced by CHIKV in C6/36 cells. However, Schnettler et al. [102] showed that transfection of dsRNA alone is not sufficient to induce production of piRNA-like molecules, thus providing evidence that ssRNA as a result of active virus replication is required, and Vodovar et al. [123] also argue that piRNAs are derived from viral ssRNA generating primary piRNAs from the (−) strand and secondary piRNAs from the abundant (+) sense mRNA. Another

observation indicating that the origin of virus-specific piRNAs is likely to be viral ssRNA of genomic or subgenomic origin is that the majority of piRNAs derived from SFV, SINV and CHIKV map to the coding strand in the 5' end of the subgenomic mRNA or to a lesser extent to the 5' end of the genomic mRNA [82, 102, 123]. The mapping of virus-derived piRNAs to specific regions in all three alphaviruses, as opposed to a more general distribution of 21 nt viRNAs across genome and antigenome [111], suggests that specific viral sequences or secondary structures are the source of virus-derived piRNAs. Future studies should elucidate how dsRNA and viral ssRNA contribute to the induction and generation of piRNAs.

In cells derived from *Culicoides* biting midges, viRNAs with PIWI signature have been observed after infection with bluetongue virus (BTV) and Schmallenberg virus (SBV) [103], suggesting this newly-discovered antiviral mechanism is not only a mosquito-specific response. Future work on other vector species including ticks will elucidate whether this mechanism has evolved in specific groups of dipteran vector species or is also an antiviral response in other arthropods.

siRNAs

While there is both an endogenous [28, 38, 91] and an exogenous [14, 99] siRNA pathway in insects, this review will only cover the exogenous pathway which is mainly associated with antiviral RNAi. There has been one report of SINV infection inducing a novel class of endogenous siRNAs, but it is not clear whether this is of benefit for the virus or the vector [1]. The exogenous siRNA pathway is considered to be the most important antiviral mechanism in arbovirus vectors such as mosquitoes [14]. The mechanism in mosquitoes is very similar to the one described for the model organism *D. melanogaster* (reviewed by [32, 34]). Upon infection, RNA viruses introduce or generate foreign dsRNA in the arthropod cell either as replication intermediates, or as part of their dsRNA genome or as secondary structures of their ssRNA genome. dsRNA is recognised as a pathogen-associated molecular pattern (PAMP) by Dicer-2 (Dcr-2). In association with R2D2, Dcr-2 cleaves dsRNA into virus-induced small interfering RNAs, designated viRNAs, usually 21 nt long. The viRNAs are then incorporated into the RNA-induced silencing complex (RISC) with Argonaute-2 (Ago-2) as its integral protein. Ago-2 is also called the Slicer protein. Slicer activity is essential for an efficient RNAi response against flaviviruses [18]. One strand, the passenger strand, of the viRNA is degraded and the RISC uses the remaining guide strand to target complementary RNA including viral mRNA and viral genomes. Upon interaction with a complementary RNA, Ago-2 slices the target. In the case of mRNA this in turn inhibits

synthesis of viral proteins. It has been suggested that Dcr-2 is also involved in signalling. Like RIG-I or Mda-5 in mammalian cells, Dcr-2 detects dsRNA as foreign and activates signalling which, at least in mosquitoes, results in increased expression of *vago*, a cytokine with an interferon-like function [92].

An important part of the RNAi response is systemic spread. Systemic RNAi and amplification of small RNAs has been shown in plants and *Caenorhabditis elegans* [113, 124, 131]. In *D. melanogaster* it has been shown that systemic spread of the RNAi response is essential for an efficient antiviral response and components of the dsRNA uptake machinery have been identified [60, 98]. To date no protein with RNA-dependent RNA polymerase (RdRP) activity for amplification of small RNAs has been identified in insects [72, 73]; however, it was proposed that reverse transcription of viral RNA by retrotransposons may result in viral cDNA elements serving as a template for the generation of new viRNAs [39]. In U4.4 cells it has been shown that siRNAs can spread through the culture if there is cell-to-cell contact, but cannot spread freely through the culture medium [6].

While RNAi in mosquitoes is relatively well characterised, little is known about RNAi in other arbovirus vectors. Despite the current lack of a published genome, RNAi has recently been shown to target replication of BTV and SBV in *Culicoides* cells in a manner similar to other arbovirus vectors [103]. RNAi has also been identified as an antiviral mechanism in tick cells [36, 37] and has been used as a tool for knockdown of gene expression in tick and tick-borne disease research for over a decade [11, 29], but the detailed mechanisms are still not well understood. For the two tick species *I. scapularis* and *R. microplus*, components of the RNAi pathway have been identified by comparative genomics [65]. For both species, putative Dcr-1, Ago-1 and Ago-2 as well as orthologues of many proteins involved in dsRNA uptake and systemic RNAi in *D. melanogaster* have been identified [65]. However, it is not known if these tick proteins exhibit the expected Dcr-1, Ago-1 and Ago-2 activity in ticks. Kurscheid et al. [65] also proposed that an RdRP responsible for amplification of the RNAi response is present in ticks. However, there is no experimental evidence for this and while the application of dsRNA for knockdown of gene expression in ticks indicates efficient systemic RNAi [29], the detailed mechanisms remain unknown.

Many insect and plant viruses have developed strategies to counteract RNAi, such as suppression and evasion of the RNAi response (reviewed by [32]). These will not be covered in this review. For a summary of known suppressors of RNAi expressed by insect and plant viruses, see http://viralzone.expasy.org/all_by_protein/891.html [77]. One interesting observation is that while some arboviruses

do have RNAi evasion strategies [111], they generally do not encode efficient suppressors of RNAi. Strong suppression of the antiviral RNAi response could be detrimental to vector survival [24, 84] and thus to the transmission of the arbovirus. The only arboviruses known to encode suppressors of RNAi are flaviviruses. WNV encodes a subgenomic RNA in the 3' untranslated region of its genome (subgenomic flaviviral RNA) which acts as an RNAi suppressor in both mammalian and insect cells [101]. The NS4B protein of another flavivirus, DENV-2, has also been shown to act as a suppressor of mammalian and insect RNAi [59].

Antiviral responses triggered by innate immunity signalling pathways

While RNAi is an important and essential component of the antiviral response, there is evidence for the involvement of several other innate immunity pathways in controlling arbovirus infections in arthropod vectors. All of the three major innate immunity signalling pathways in insects, namely Janus kinase/signal transducer and activator of transcription (JAK/STAT), Toll and Immune deficiency (Imd), have been implicated in antiviral defences in insects (reviewed by [34, 62, 81]). All three pathways also play a role in development and especially in immune responses to other pathogens. These pathways are very well-studied in *Drosophila*, but less so in arbovirus vectors. In mosquitoes it is largely assumed that mechanisms are very similar to those in *Drosophila* and components of the pathways are found in the published mosquito genomes. A comparative genomic study of *D. melanogaster*, *A. gambiae* and *A. aegypti* examined the evolutionary dynamics of immunity genes in higher insects [126]. In ticks, knowledge about immunity pathways is generally very limited; however, immune responses against bacteria have been studied in some detail [63, 74] and the knowledge gained about bacterial defences may provide a basis for studies on antiviral defence mechanisms.

The JAK/STAT pathway

Among arbovirus vectors, the JAK/STAT pathway and its antiviral activity have been characterised predominantly in mosquitoes. JAK/STAT pathway activation inhibits replication of the flaviviruses DENV and WNV in, respectively, *A. aegypti* mosquitoes [115] and *C. quinquefasciatus* Hsu cells [92]. Upon flavivirus infection, mosquito Dcr-2 recognises viral dsRNA in a similar manner to RIG-I and Mda-5 in mammals and initiates a signalling cascade leading to the expression and secretion of Vago [92], a small cytokine with a function similar to interferon in the

mammalian system. Secreted Vago binds to an unknown receptor on neighbouring cells leading to the signalling cascade shown in Fig. 1 and resulting in activation and

translocation of STAT. Activated STAT acts as a transcription factor inducing expression of antimicrobial peptides (AMPs) such as two DENV restriction factors [115]

and vir-1 [92]. However, treatment with poly I:C (a synthetic analogue of dsRNA) or BTV dsRNA did not lead to *vago* upregulation and STAT activation in *C. quinquefasciatus* cells [92], and infection of U4.4 cells with the alphavirus SFV did not lead to activation of STAT [33]. Taken together, these findings suggest that activation of STAT may be a specific anti-flaviviral response. However, replication of SFV was reduced when STAT was activated prior to SFV infection by treatment with heat-inactivated *Escherichia coli* bacteria [33]. Due to the concurrent activation of the Imd pathway, it could not be concluded if this was a specific consequence of JAK/STAT activation. However, alphavirus replication does appear to induce activation of STAT in *D. melanogaster*. Replication of SINV in *D. melanogaster* resulted in increased expression of *vago* and *attacin-C* (*attC*), among other STAT-regulated genes. Furthermore, *attC* was shown to have an antiviral function against SINV in this system [50]. In another study the effect of SINV infection on gene expression levels of *stat* in Aag2 cells was investigated. While there was no direct evidence of STAT activation, *stat* expression levels increased approximately 1.5-fold upon SINV infection [9].

In other arbovirus vectors such as ticks and midges there is only limited information on JAK/STAT. In *I. scapularis* ticks it was shown that there is a functional JAK/STAT pathway which controls infection with the intracellular tick-borne bacterium *Anaplasma phagocytophilum* [74], but some of the key players of the pathway remain unknown and there is no information on whether JAK/STAT may also have an antiviral function. New insights into the role of JAK/STAT in the antiviral response of midges may be gained in the future through analysis of the *Culicoides* genome.

The Toll pathway

Unlike the mammalian Toll-like receptors (TLRs), arthropod Toll receptors are not pattern recognition receptors (PRRs); pattern recognition in arthropods is mediated by extracellular soluble PRRs [64, 70]. Upon recognition of PAMPs, for example lipoteichoic acid of gram-positive bacteria, fungal peptidoglycan and possibly virus debris, the cytokine spaetzle is cleaved into its active form and binds to the Toll receptor [4]. A downstream signalling cascade similar to those of mammalian TLRs results in degradation of the I κ B orthologue Cactus and activation of the transcription factor REL1 [108], an orthologue of NF κ B, which translocates to the nucleus and initiates transcription of AMPs such as defensins. During DENV infection of *A. aegypti* mosquitoes the Toll pathway is activated in the midgut and plays an important role in controlling DENV infection [94, 130]. In contrast to DENV, evidence so far suggests that alphaviruses are not controlled by Toll in insects. In U4.4

cells SFV replication was not reduced by expression of a constitutively active Toll receptor; in fact there was a small increase in replication [35]. In *Drosophila* SINV was unaffected by mutations in the Toll pathway [8]. However, both systems used in these experiments with alphaviruses were not natural whole mosquito infections. While U4.4 cells are derived from a biologically relevant mosquito (*A. albopictus*), cell lines do not represent all cell phenotypes and cannot reproduce events in a complex environment such as the midgut. The study performed with SINV in *Drosophila* used whole flies; however, *Drosophila* is not a blood-feeding vector of arboviruses and thus may lack mechanisms evolved specifically against vector-borne alphaviruses. In *A. gambiae* concurrent injection of ONNV and dsRNA against Cactus resulted in an increased viral load 7 days post-injection compared to dsRNA controls which did not target Cactus [125]. If the Toll pathway was involved in controlling alphavirus replication in mosquitoes, knocking down expression of *cactus*, as a negative regulator of the pathway, should result in a decrease of virus replication. Thus these results provide further evidence that the Toll pathway is not involved in antiviral responses to alphavirus replication.

In ticks and midges the Toll pathway has not been characterised and components are mostly unknown. However, for *I. scapularis* there are a number of putative Toll receptors in the published genome [46] and also orthologues of REL1 and Cactus [63]. Their role in immune defences against bacterial or viral infection remains unknown.

The Imd pathway

The Imd pathway in mosquitoes is known to be activated by gram-negative bacteria. A downstream signalling cascade [62, 81] (Fig. 1) results in activation of REL2 [106], another orthologue of NF κ B. The Imd pathway in *Drosophila* has been implicated in the antiviral response to insect viruses such as cricket paralysis virus [27], sigma virus [119] and Nora virus [26]; however, the only arboviruses known to be associated with an Imd immune response in insects are alphaviruses. SINV replication in *D. melanogaster* resulted in increased expression of components of the Imd pathway and the downstream gene *dip-tericin-B* has antiviral activity against SINV [50]. Pre-activation of the Imd pathway by heat-inactivated *E. coli* also increased SFV replication in U4.4 cells [33]. In the mosquito *A. gambiae*, ONNV infection led to changes in expression of Imd pathway components, possibly activating the Imd pathway, but since knockdown of REL2 gene expression did not affect virus production, the role of Imd in ONNV infection remains unclear [125].

While orthologues of some components of the Imd pathway such as REL2 and the negative regulator Caspar can be found in the *I. scapularis* genome, a tick orthologue

of Imd itself could not be identified by basic local alignment search (tblastn; authors' unpublished findings). NF- κ B orthologues named RelA and RelB were characterised in nuclear extracts of the *I. scapularis* cell line ISE6 and were found to act as transcription factors with binding sites similar to those of other organisms [87]. These authors also showed that binding activity of RelA and RelB was increased during infection with *A. phagocytophilum* indicating activation of an Imd-related pathway by this intracellular bacterium. Whether this pathway plays a role in virus infection of ticks remains unknown. In addition, a relatively large number of AMPs have been found in ticks and some of them, such as defensins, have been well characterised; however, their regulation remains unclear and, in particular, nothing is known about their antiviral effects (reviewed by [63]).

Other antiviral defences of arthropods

Besides RNAi and signalling pathways there are many other responses in arthropods to virus infection including melanisation [97] and cell stress responses such as autophagy and regulation of heat shock proteins (reviewed by [62, 81]). However, since heat-shock proteins are important for loading siRNA into the RISC in *Drosophila* [57], upregulation of heat-shock proteins may not be an antiviral response in itself, but a necessary step for efficient antiviral RNAi.

Melanisation is a wound healing process, which is also used to defend against bacteria in insects. It is a humoral response mediated by a cascade of molecules and enzymes in the haemolymph, involving the key enzyme phenoloxidase (PO), and is often referred to as the PO cascade [16, 22]. A decade ago it was shown that expression of dsRNA targeting a precursor of PO (pro-phenoloxidase I) by SINV lowered PO activity in the mosquito *Armigeres subalbatus* and increased SINV replication [116]. However, this has been followed up only recently and PO activity has been shown to act as an antiviral response to SFV in *Aedes* mosquitoes [97]. SFV was able to activate PO in U4.4 cell-conditioned medium with similar efficiency to *E. coli* and, when a suppressor of the PO pathway was inserted into the SFV genome, virus replication was increased in *A. aegypti* mosquitoes [97]. Another study showed that ONNV infection of *A. gambiae* reduced expression of components of the melanisation cascade and in co-infection studies ONNV infection inhibited melanisation of *Plasmodium* ookinetes [125]. Thus it is possible that *A. gambiae* PO has antiviral activity and that ONNV has evolved to counteract this mechanism.

In ticks melanisation is a controversial topic and it remains unclear whether ticks have a PO cascade. PO activity has been identified in horseshoe crabs [85, 88],

which as ancient arachnids are more closely related to ticks than are insects. There has been one report of PO activity in a nymphal stage of the argasid tick *Ornithodoros moubata* [58], while another study found no evidence of PO activity in three ixodid tick species of different genera [134]. The latter result may have been due to the small amounts of haemolymph used or due to a PO inhibitory factor present in the samples. However, no orthologue of PO could be found in the *I. scapularis* genome [63] indicating that there is a possibility that ixodid ticks have lost this mechanism (argasid ticks are ancestral to ixodid ticks) or that a different protein may have evolved to have a comparable function to PO.

Concluding remarks

Our understanding of antiviral responses in arthropod vectors has increased immensely over the last decade. Insights into antiviral responses in the model insect *Drosophila* and high throughput sequencing studies have helped to identify antiviral mechanisms in arthropod vectors. While the most important antiviral mechanism is still considered to be exogenous RNAi, it is becoming more evident that the innate immune system of arthropods is highly complex and that other very specific mechanisms have evolved to defend against different viruses. In fact, evidence suggests that there are significant differences between the different arbovirus families which need to be taken into consideration. Some (or possibly all) flaviviruses have the ability to suppress RNAi [59, 101]; thus it is not surprising that other innate immunity pathways such as JAK/STAT and Toll are involved in controlling flavivirus infection in mosquitoes [92, 115, 130]. In contrast, alphaviruses are not known to actively suppress RNAi and use only decoy mechanisms to evade the RNAi response [111]. It is possible that antiviral RNAi is thus more efficient in controlling alphavirus replication and innate immunity signalling pathways may play only a small role in controlling alphavirus replication in mosquitoes. There is still very little known about how other arboviruses such as bunyaviruses or reoviruses are controlled by their arthropod vectors. The different replication strategies of these viruses may require different control mechanisms. The recent discovery of putative new antiviral responses such as melanisation and, particularly, the implication of piRNAs in mosquito antiviral immunity against a variety of arboviruses (CHIKV, SFV, SINV, DENV, LACV, RVFV, SBV and BTV) [45, 68, 82, 102, 103, 123], show how complex the arthropod immune system is and how far there is still to go in understanding it. One of the questions that will surely be addressed in the future is how virus-derived piRNAs are generated and whether it is possible to use the

knowledge gained to benefit vector control strategies. There may still be components of the immune system that we are largely unaware of, as the recent implication of Down syndrome cell adhesion molecule (Dscam) in antiviral immunity in crustaceans has shown [20]. Dscam has a hypervariable domain and may act as a mediator of adaptive immunity in arthropods.

Understanding of vector immunity and also of interactions between multiple co-infecting microorganisms may lead to new strategies to control vectors of arboviruses and limit their vector capacity or competence. The introduction of a strain of the intracellular bacterium *Wolbachia* into *A. aegypti* mosquitoes, for instance, interferes with many aspects of *A. aegypti* as an arbovirus vector including reduced fitness and a reduction in DENV replication [56]. This interplay of microorganisms shows the importance of comprehensively understanding the arthropod immune system to exploit such approaches for future vector control strategies.

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