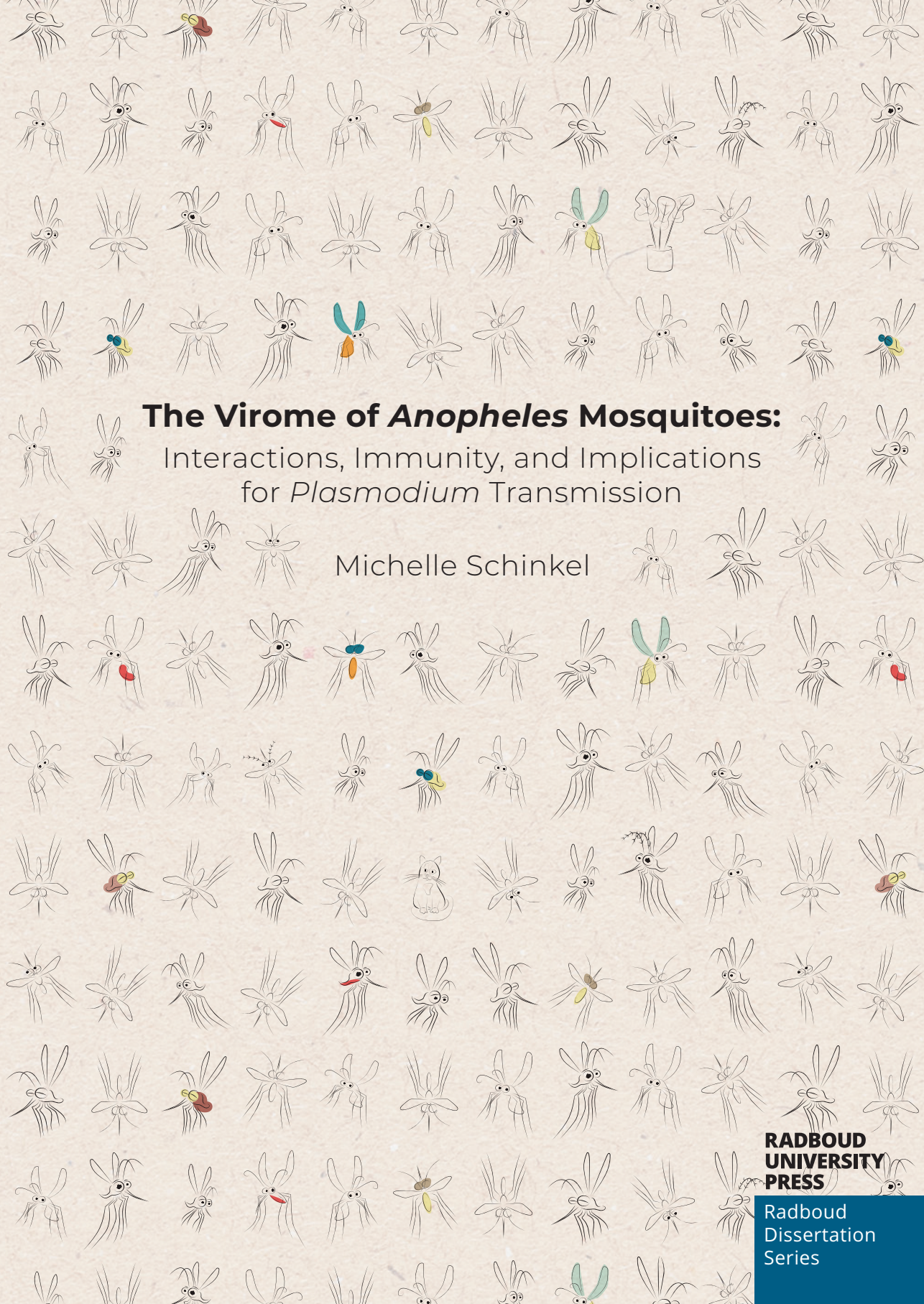




The Virome of *Anopheles* Mosquitoes:

Interactions, Immunity, and Implications
for *Plasmodium* Transmission

Michelle Schinkel

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Plasmodium Transmission

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Interactions, Immunity, and Implications for
Plasmodium Transmission

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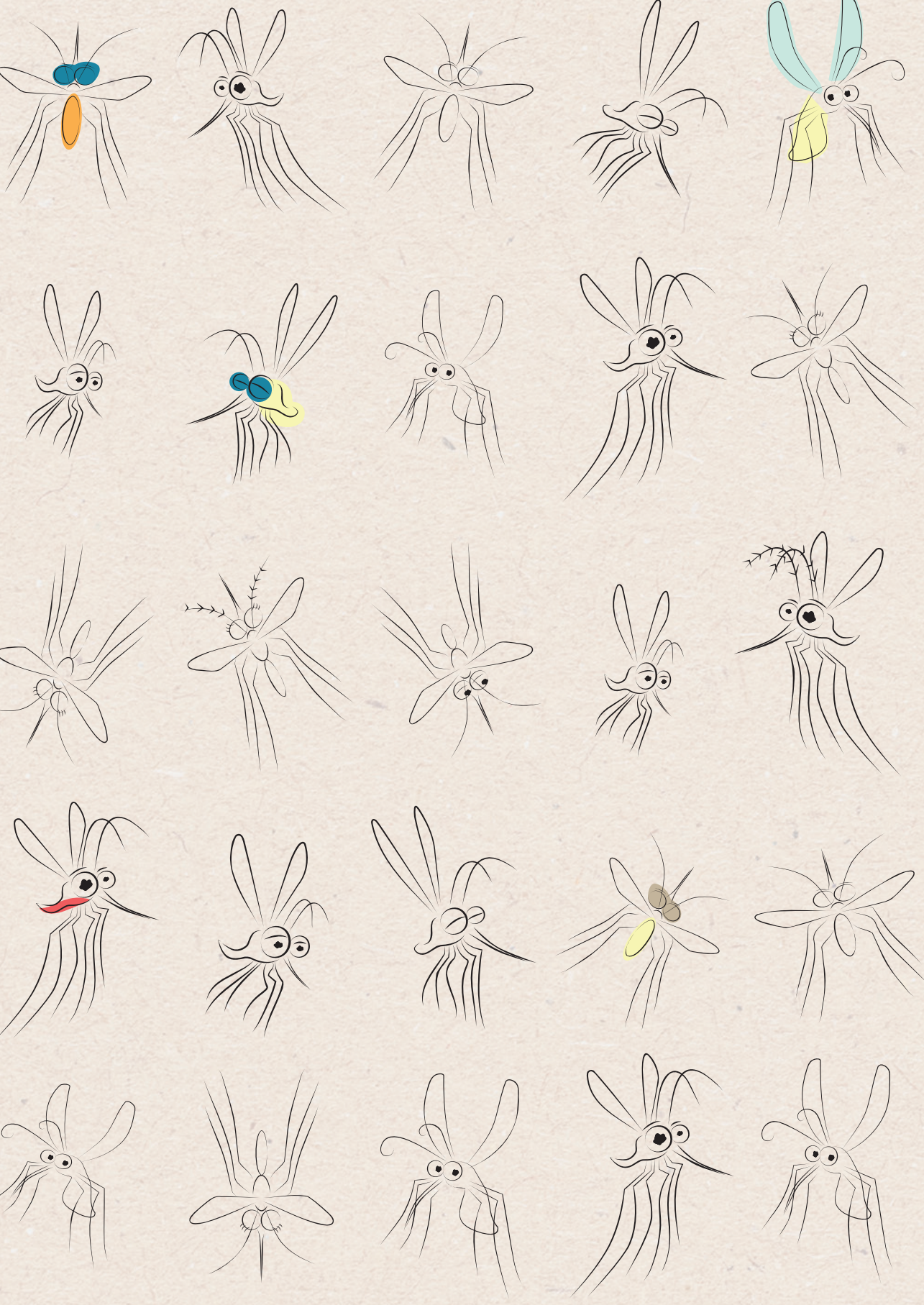
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CHAPTER 1

General introduction and outline of the thesis: Tripartite interactions between viruses, parasites, and mosquitoes

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Abstract

Mosquito-borne diseases have a major impact on global human health. Biological agents that colonize the mosquito vector are increasingly explored as an intervention strategy to prevent vector-borne disease transmission. For instance, the release of mosquitoes carrying the endosymbiotic bacterium *Wolbachia* effectively reduced dengue virus incidence and disease. Insect-specific viruses, which infect mosquitoes but are not transmitted to vertebrate hosts, are likewise considered as biocontrol agents against vector-borne diseases. While most studies focused on insect-specific viruses as an intervention against arboviruses, we here consider whether mosquito-specific viruses may affect transmission of the malaria causing *Plasmodium* parasite by *Anopheles* mosquitoes. While there is no direct experimental evidence addressing this question, we found that viral infections of dipteran insects activate some of the immune pathways that are antiparasitic in *Anopheles*. While these findings suggest that insect-specific viruses may modulate malaria transmission, the tripartite interactions between viruses, parasites, and *Anopheles* mosquitoes remain largely unstudied.

Introduction

The parasite *Plasmodium falciparum*, transmitted by *Anopheles* mosquitoes, is responsible for over 240 million clinical malaria cases and approximately 600,000 deaths each year. In addition to this direct health burden, malaria has a major economic impact with direct government spending of over 4.5 billion USD per year and indirect costs accounting for up to 1.3% loss in gross domestic product (GDP) growth in malaria-endemic countries [1, 2]. Insecticides and antimalarial drugs have effectively reduced the disease burden [3], but their success is threatened by mosquito resistance to insecticides [4] and parasite resistance to artemisinins [5, 6]. Moreover, recently approved vaccines only provide incomplete protection [7, 8], necessitating the development of complementary approaches for malaria control.

Symbiotic microbes that colonize the vector provide opportunities to fight mosquito-borne disease transmission (reviewed in [9]). The potential of this approach is probably best illustrated by the strong reduction in dengue virus infection incidence and hospitalization in regions in which *Aedes aegypti* mosquitoes carrying the endosymbiotic bacterium *Wolbachia pipientis* were released [10]. Biological agents are likewise considered for malaria control. For example, the bacterium *Deltia tsuruhatensis* TC1 produces an alkaloid compound, called harmaline, that disrupts the development of female gametes in the mosquito gut. Accordingly, *Deltia* colonized mosquitoes produced approximately 75% fewer oocysts than control mosquitoes lacking the bacterium and inefficiently transmitted *Plasmodium* to mice [11]. Interestingly, the bacterium could be administered as a suspension in sugar water in semi-field conditions, suggesting a cost-effective manner to introduce the bacterium into wild mosquito populations. Similarly, apparently non-pathogenic and vertically transmitted *Microsporidia* MB obtained from field populations of *An. arabiensis* mosquitoes in Kenya, result in high levels of impairment of *P. falciparum* transmission [12]. Another microbe-based approach involves the use of symbiotic bacteria, such as *Serratia* strain AS1 or *Pantoea agglomerans*, to drive expression of anti-malarial effector proteins in the mosquito midgut (referred to as paratransgenesis), which effectively reduced infection intensity (oocyst numbers) in mosquitoes [13, 14]. However, the release of genetically modified bacteria into the ecosystem may pose hurdles for regulatory approval and public acceptance of paratransgenesis-based approaches.

Recent metagenomic studies indicate that mosquitoes carry a large reservoir of insect-specific viruses (ISVs) [15], which may affect transmission of vector-borne pathogens. Interference of ISVs with arthropod-borne (arbo-) virus replication has already been studied for several virus-mosquito combinations *in vitro* and *in vivo*.

A special case of interference is superinfection exclusion, where a pre-existing infection prevents a subsequent infection with the same or a closely related virus. For example, superinfection exclusion has been observed in *Aedes aegypti* where the insect-specific alphavirus Eilat virus reduced replication of several arthropod-borne alphaviruses (Sindbis virus, chikungunya virus, and the Venezuelan-, Eastern-, and Western equine encephalitis viruses) in cell culture and chikungunya virus in adult mosquitoes [16]. Likewise, the insect specific flavivirus Palm Creek virus suppressed replication of the West Nile and Murray Valley encephalitis viruses [17], and Nhimirim virus (*Flavivirus* genus) reduced replication of West Nile virus, Japanese encephalitis virus, and St Louis encephalitis virus in an *Aedes albopictus* cell line [18]. *In vivo*, coinfection of Nhimirim virus resulted in a lower percentage of West Nile virus positive saliva samples in *Culex quinquefasciatus* mosquitoes, but, unexpectedly, not in *Culex pipiens* [19]. However, ISV interference with arbovirus replication is not restricted to related viruses as the insect virus Phasi Charoen-like virus (*Phasivirus* genus, *Phenuiviridae* family) suppressed replication of La Crosse virus (*Orthobunyavirus* genus, *Phenuiviridae* family) as well as the unrelated arthropod-borne flaviviruses Zika and dengue virus in *Ae. albopictus* cells [16]. The mechanisms by which ISVs interfere with arbovirus replication remain poorly understood, and whether ISVs affect transmission of non-viral pathogens has not been studied.

Box 1. Literature search strategy

A two-step search strategy was used to identify antiparasitic immune responses that may be affected by viral infection in mosquitoes. In the first step, a PubMed search was performed (on 18-01-2024) using the terms (((((plasmodium[MeSH Terms]) OR (malaria[MeSH Terms])) AND (immunity[MeSH Terms])) AND (Anopheles[MeSH Terms]) NOT (mammalian[MeSH Terms])) NOT (avian[MeSH Terms])). Hits were evaluated manually to identify *Anopheles* immune factors and pathways shown to affect parasite growth. In the second step, PubMed searches were performed (between 07-03-2024 and 19-03-2024) to identify papers showing that these immune pathways were activated or modulated by viral infection. Given the sparsity of data about interactions between viruses and the *Anopheles* immune response, we broadened our search to the entire order of Diptera, using the MeSH terms Diptera and Viruses in combination with the highest node in the MeSH tree covering entire immune pathways (MeSH terms Hemocytes, Reactive Oxygen species, JNK pathway, Complement, Toll-like Receptor, IMD proteins, Janus kinase, Melanin, Fibrinogen). We considered all viral infections, irrespective of their transmission mode (vector-borne or insect-specific).

In this review, we explore whether ISVs have potential as a biological control agent against malaria transmission by *Anopheles* mosquitoes, under the hypothesis that ISVs may prime mosquito immune pathways with antiparasitic activity. We first provide a brief overview of the antiparasitic immune responses of the *Anopheles* vector against *Plasmodium* (for more comprehensive reviews, see [20–22]). We next performed a literature search for experimental support that the identified antimalarial pathways are activated or modulated by viral infection (the literature search strategy is described in Box 1). We end the review with a synthesis in which we speculate whether ISVs affect malaria transmission under natural conditions or can be considered as an intervention strategy.

Malaria life cycle and *Anopheles* immune responses

Malaria transmission to mosquitoes starts with the formation of specialized sexual stage parasites (male and female gametocytes) that, for the deadliest malaria parasite *P. falciparum*, appear in the blood stream of an infected person at approximately 9–12 days after the first detection of asexual parasites [23]. These gametocytes can be ingested through blood feeding by female *Anopheles* mosquitoes where they activate and fertilize to form elongated and motile ookinetes that penetrate the midgut wall to form oocysts, in which several rounds of nuclear division occur. These oocysts enlarge over time and release sporozoites that invade the salivary glands and render the mosquito infectious to humans upon their next bite. Only a small proportion of the gametocytes that are initially taken up with the blood meal successfully complete the transmission cycle and penetrate the salivary glands as sporozoites to infect the next vertebrate host. This depends on the molecular interactions between the two species, especially the mosquito immune response that plays a pivotal role in recognizing and eliminating the invading pathogen (Figure 1). The mosquito immune response to *Plasmodium* consists of a humoral immune response, mediated by a complement-like system and the melanization cascade, and a cellular immune response, mediated by hemocytes that phagocytose and encapsulate pathogens and produce essential molecules for the humoral response [20–22].

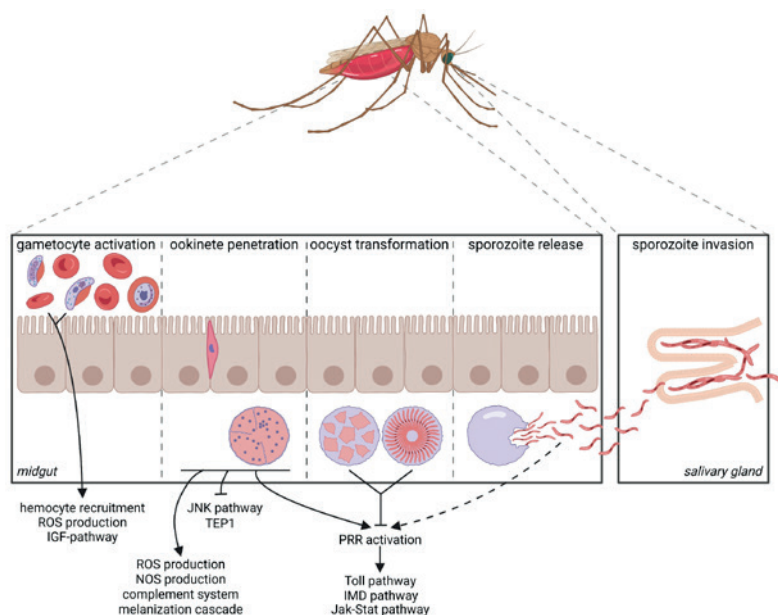


Figure 1. Malaria life cycle and immune responses induced in *Anopheles*. Ingestion of male and female gametocytes and red blood cells in the midgut lumen triggers the recruitment of hemocytes, induces production of reactive oxygen species (ROS), and activates the insulin-like growth factor (IGF) pathway. Upon sensing a temperature drop of approximately 5 °C, an increase in extracellular pH, and the presence of xanthurenic acid, the gametocytes fuse to form a zygote (fertilization) [7]. After fertilization, the zygote undergoes meiosis and develops into an ookinete, which perturbs the midgut cell layer and translocates to the basal laminal side. By doing so, the ookinete causes midgut cell damage which induces nitric oxide synthase (NOS) and ROS expression. The ookinete rounds up to form the nonmotile oocyst, which initiates sporozoite formation (sporogony). Membrane proteins on the ookinete's surface suppress the activation of immune pathways including the JNK pathway and the complement TEP-1 protein. Around two weeks after blood meal ingestion, the oocyst is mature and bursts, releasing sporozoites into the hemocoel, the mosquito's open circulatory system, to migrate to the salivary gland, where they can be transmitted to a naïve mammalian host. Approximately 10–20% of the sporozoites invade the salivary gland, suggesting efficient immune mechanism that eliminate or lyse sporozoites. Relevant immune pathways are indicated below the figure; see text for details.

Gametocyte activation and midgut responses

Blood feeding induces proliferation and activation of hemocytes, leading to higher expression of immune factors, including proteins essential for the complement system (such as thioester containing protein 1, TEP1) and melanization (such as prophenoloxidase, PPO) [24–26]. Blood feeding also increases reactive oxygen species (ROS) expression in the midgut and fat body, which is further enhanced locally in the midgut as demonstrated for *Plasmodium berghei* infected mosquitoes due to a reduced expression of detoxifying proteins such as catalase, resulting in high hydrogen peroxide levels that induces parasite lysis [27]. Human insulin-like growth factors

(IGF), ingested along with the blood meal, activate the insulin signaling pathway in midgut epithelial cells leading to an Akt mediated decrease in the mosquito lifespan, limiting the time window for successful parasite transmission. Moreover, experiments using transgenic mosquitoes suggest that Akt activation directly impacts *Plasmodium* development in the mosquito midgut, resulting in low infection rates and parasite load [28, 29]. Inside the midgut lumen, the parasite may compete with midgut-resident microbiota, resulting in reduced expression of genes encoding antimicrobial peptides (AMPs) such as cecropins, defensins and gambicin [30]. Moreover, elimination of the gut microbiota increased mosquito susceptibility to *Plasmodium* infection, and, conversely, co-feeding *Plasmodium* with bacteria reduced parasite infection levels, likely by activating the mosquito immune response [31].

Ookinete penetration, complement, and melanization

Approximately one day after an infectious blood meal, ookinetes penetrate the peritrophic matrix lining the midgut lumen and invade and traverse the midgut epithelium. At this moment, gut resident bacteria are exposed to the midgut epithelium, which initiates an antibacterial response and triggers the release of a hemocyte differentiation factor from the fat body, resulting in increasing numbers of circulating hemocytes [32]. During midgut traversal, there is no direct contact of parasites with hemocytes, but parasites are exposed to soluble factors in the hemolymph and, thus, to the humoral immune response of the mosquito. Ookinete penetration of the midgut wall represents the biggest hurdle in the mosquito life cycle of the plasmodium parasite, as approximately 80% of the invading ookinetes are killed by the mosquito immune system [33], predominantly the complement-like system (reviewed in [20]).

Parasite invasion causes cell death of midgut cells and induces the expression of genes encoding nitric oxide synthase (NOS), which produces nitric oxide, and heme peroxidase (HPX2), NADPH oxidase 5 (NOX5), and dual oxidase (Duox) that produce hydrogen peroxide [34, 35]. Nitric oxide can react with hydrogen peroxide to produce reactive nitrogen species that mediate protein nitration, which marks the ookinete surface and acts as a recognition signal for the mosquito complement-like system [34, 36, 37]. Thioester-containing proteins (TEP)-1, a homolog of the mammalian complement factor C3, along with the leucin-rich repeat proteins APL1C and LRIM1, binds and lyses the nitrated ookinete [34, 38].

Expression of *HPX2*, *NOX5*, *TEP1* (essential for the complement-like system) and *FBN9* (see below) are regulated by the Jun-N-kinase (JNK) pathway, a pathway essential for wound healing in *Drosophila*. Membrane proteins on the ookinete, such as Pfs47 and

PIIMS43 (Plasmodium Infection of the Mosquito Midgut Screen 43) suppressing JNK signaling in the midgut, thereby suppressing the nitration reaction and complement mediated parasite killing [37, 39-41]. Accordingly, polymorphisms in TEP1 and Pfs47 affect the efficiency of the complement-mediated response [42, 43].

The complement system is tightly linked to the melanization cascade that deposits melanin at wound sites or around pathogens. Pathogen recognition activates this pathway via the activation of CLIP-domain serine proteases that cleave prophenoloxidasases (PPOs) to release the active phenoloxidase enzyme that produce melanin precursors. Multiple CLIP proteases are involved in regulating the melanization response to *Plasmodium*, with potential overlap and synergy between them [44, 45]. Depending on the mosquito genetic background, melanization may be involved in direct ookinete killing or in disposing of parasites that were killed by the complement system [33, 45].

Immune signaling cascades and pattern recognition receptors

Immune signaling pathways are essential for the initiation and coordination of the humoral response and the expression of immune effectors. Three pathways are involved in antimalarial defense in *Anopheles*, the Jak-Stat pathway and two NF- κ B transcription factors regulated immune signaling pathway, the Toll pathway that activates the REL1 transcription factor and the IMD pathway that activates the REL2 transcription factor [21, 22]. Activation of the Toll and IMD pathways results in REL1 and REL2 translocation to the nucleus, leading to the production of antimicrobial peptides (AMPs) such as defensins, cecropins, attacin, and gambicin that neutralize pathogens [31]. In line with this, transgenic expression of Cecropin A behind the blood meal inducible carboxypeptidase promoter significantly reduced *P. berghei* oocyst numbers in the *An. gambiae* midgut [46, 47]. Moreover, activation of the Toll pathway (by silencing of the negative regulator *Cactus*) increased the number of oenocytoids, a population of secretory immune cells that produce soluble factors involved in the complement-like system [48]. The STAT transcription factor regulates expression of NOS and *TEP1* and is, thus, critical for the antiplasmodial response in *An. gambiae* [49]. Unexpectedly, the relative importance of Toll and IMD pathways seems to differ between *Plasmodium* species, as the Toll pathway seems more active against the rodent malaria parasite *P. berghei* and the IMD pathway more active against the human malaria parasite *P. falciparum* [50-52].

The Toll and IMD pathways are activated by the recognition of pathogen-associated molecular patterns on the parasite surface by humoral pattern recognition receptors (PRRs) [53]. The Toll pathway is activated by humoral receptors such as peptidoglycan recognition proteins (PGRPs) and gram-negative binding protein that activates

a cascade of CLIP proteases eventually leading to the cleavage of proSpaetzle to Spaetzle, the active Toll receptor ligand. The IMD pathway, on the other hand, is activated more directly by PRRs such as PGRPs. Another important class of PRRs are members of the fibrinogen-related protein family (FREP, also referred to as fibrinogen domain immunorecognition, FBN) [54]. For example, the JNK-regulated FBN-9 was found to strongly co-localize with *P. berghei* and *P. falciparum* and mediate antiparasitic defense [54, 55].

Oocyst transformation and sporozoite invasion

Upon reaching the basal side of the midgut, ookinetes undergo physiological changes and transform into oocysts that lose mobility and remain embedded between the epithelium and the basal lamina of the midgut. Ookinete binding to components of the basal lamina, such as collagen IV and laminin, is thought to trigger ookinete to oocyst transformation [56, 57]. A thick capsule forms that protects the oocyte and shields it from recognition by the mosquito immune system, possibly by the integration of mosquito ‘self proteins’ [57]. Interestingly, parasites lacking the capsule protein Cap380 form oocysts in normal numbers, but are gradually eliminated before completing maturation, possibly due to immune clearance [58, 59]. Around two weeks after an infectious blood meal, oocysts rupture and sporozoites are released into the hemocoel. Salivary gland invasion of these released sporozoites is only successful for 10–20% of the sporozoites, suggesting an effective immune response to phagocytosis, lysis, or clearance of sporozoites in the hemocoel [60]. Successful salivary gland invasion seems to rely on the parasite’s ability to evade mosquito immunity by posttranslational modifications of the circumsporozoite protein (CSP), a highly abundant protein on the sporozoite surface. This modification of glutamine or glutamic acid residues into pyroglutamic acids is mediated by the parasite’s glutaminyl cyclase (QC), and oocysts and sporozoites lacking the QC enzyme or carrying an alanine substitution of the target glutamine became sensitive to mosquito melanization and phagocytosis by hemocytes [61].

***Anopheles* viruses and antiviral immunity**

While *Anopheles* mosquitoes are best known for their role in malaria transmission, they are also host to a diverse set of insect-specific viruses (reviewed in [15, 62]), including viruses from taxa that contain arboviruses, such as Beaumont virus and Wuhan mosquito virus 9 (*Rhabdoviridae*), *Anopheles* flavivirus and Quang binh virus (*Flaviviridae*), as well as viruses from taxa solely containing ISVs. The *Anopheles* virome comprises RNA viruses with positive-sense and negative-sense genomes as

well as dsRNA viruses and a DNA virus [15, 63]. While sampling is too sparse to assess the prevalence of specific viruses in local mosquito populations, multiple *Anopheles* viruses have been detected in multiple continents, at least suggesting a broad distribution across mosquito populations.

Table 1. Antiparasitic immune pathways and examples of their activation or modulation by viral infection in dipteran insects

Immune factor or pathway	Effect in viral infection	Virus	Host	Reference
Hemocytes	Increased hemocyte recruitment to midgut	Dengue and Zika virus	<i>Ae. aegypti</i>	[67]
	Infection of hemocytes	Sindbis virus	<i>Aedes</i> and <i>Culex</i> mosquitoes	[70]
	Depletion of hemocytes	Cricket paralysis virus	<i>D. melanogaster</i>	[69]
Reactive oxygen species	Induction of ROS related genes	Dengue virus	<i>Ae. aegypti</i>	[71]
Complement-like pathway	Induction of thioester containing protein-20	Chikungunya, dengue and Zika virus	<i>Ae. aegypti</i>	[73]
Melanization response	Activation of phenoloxidase cascade	Semliki Forest virus	<i>Aedes</i> mosquitoes	[76]
Jak-Stat pathway	Pathway activation	Drosophila C virus, cricket paralysis virus, IIV-6	<i>D. melanogaster</i>	[72, 85, 86]
		Dengue virus	<i>Ae. aegypti</i>	[88]
Toll pathway	Pathway activation	Dengue virus	<i>Ae. aegypti</i>	[79, 80]
	Pathway suppression	IIV-6 and Kallithea virus	<i>D. melanogaster</i>	[91, 92]
IMD pathway	Relish activation	Sindbis virus	<i>Ae. albopictus</i> cells	[82]
	Pathway suppression	IIV-6	<i>D. melanogaster</i>	[91]
JNK signaling pathway	Pathway activation	Chikungunya, dengue and Zika virus	<i>Ae. aegypti</i>	[73]

Antiviral defense in insects is mediated by a combination of small RNA pathways and transcriptionally induced responses [64]. Below, we review the literature for evidence of viral activation or modulation of insect immune pathways known to affect *Plasmodium* transmission. With exceptions (e.g., [65, 66]), interactions between viruses and the *Anopheles* immune response are hardly studied. Given this sparsity of data, we discuss these interactions for entire mosquito family (*Culicidae*) and even broaden our discussion towards the entire order of Diptera if our literature search yielded too few results (Box 1, Table 1).

Hemocytes, complement and melanization

Hemocytes phagocytose and encapsulate pathogens, produce ROS, and express essential molecules for melanization (such as prophenoloxidase) and complement-like pathways (such as TEP-1) that are crucial for mosquito antimalarial immunity. Hemocytes are recruited to the midgut by blood feeding; this recruitment is further enhanced by infection with dengue and Zika virus in *Ae. aegypti*, suggesting that hemocytes are activated and engage in antiviral defense [67]. Indeed, hemocytes are capable of engulfing virally-infected cells in the fruit fly *Drosophila melanogaster* [68] and blocking phagocytosis or genetic ablation of hemocytes increased replication of three out of six tested viruses. In addition to the effects on hemocyte activation, viral infection may impact hemocytes more directly. For example, hemocytes were depleted in fruit flies infected with cricket paralysis virus, likely by direct infection and killing of these cells [69]. Likewise, at least one arbovirus (Sindbis virus) has been shown to infect hemocytes in four mosquito species (*Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus*, and *Cx. pipiens*) [70], likely affecting the cellular and humoral immune of the mosquito.

There is also evidence that viral infection modulates ROS production. For example, dengue virus infection induced the expression of ROS related genes in the midgut of *Ae. aegypti* at 1 day after an infectious blood meal [71], including genes involved in ROS production such as NOX5 and DUOX and peroxidase genes involved in reduction of H_2O_2 to H_2O . In *Drosophila*, infection with the DNA virus invertebrate iridescent virus 6 (IIV6) activated the NADPH oxidase NOX, likely resulting in increased ROS production, to activate the p38b MAP kinase, leading to the production of unpaired cytokines that activate the protective JAK-STAT pathway [72].

Some evidence suggests the involvement of complement like proteins in antiviral responses. Specifically, oral infection of *Ae. aegypti* with chikungunya, dengue and Zika virus resulted in the upregulation of genes encoding core JNK core pathway components, such as *Kayak* and *Puckered*. In turn, *Kayak* regulated the expression of the complement factor thioester containing protein-20 (TEP20), which was proposed to mediate antiviral activity by inducing AMP expression [73]. Likewise, the *Ae. aegypti* macroglobulin complement-related factor (AaMCR), a member of a subfamily of the insect TEP proteins, along with scavenger receptor C, was shown to bind dengue virus to activate the expression of AMPs that exert antiviral activity against dengue virus [74]. Although the phenoloxidase/melanization pathway is known to be activated by baculovirus infection of lepidoptera (e.g., [75]), only a single paper has directly studied this in mosquitoes [76]. Semliki Forest virus was shown to activate the phenoloxidase cascade in an *Ae. albopictus* cell line, and, more

importantly, infection with recombinant SFV expressing a pathway inhibitor (from *Microplitis demolitor* bracovirus) enhanced viral replication and mosquito mortality *in vivo*, suggesting that the melanization cascade is activated upon viral infection and mediates antiviral defense [76].

Immune signaling cascades

The NF- κ B related Toll and IMD pathways and the Jak-Stat pathway induce gene expression upon infection and stress. Best known for their role in the response to bacteria and fungi, increasing evidence suggests their additional involvement in the defense against several viral infections (reviewed in [64, 77]). For example, the Toll pathway is activated in response to arbovirus infection in *Ae. aegypti*, which was proposed to be mediated by sensing of double-stranded RNA by the Toll 6 receptor [78]. Indeed, expression of Toll (and Jak-Stat) pathway genes were induced after a dengue virus blood meal in *Ae. aegypti* and genetic inactivation of the Toll pathway increased virus replication [79, 80]. Likewise, the Toll pathway is activated by and mediates defense against several RNA viruses in *Drosophila* [81], and IMD pathway mutants supported higher level of Sindbis virus and cricket paralysis virus replication, resulting in higher mortality in the latter case [69, 82]. Interestingly, the IMD pathway transcription factor Relish has recently been shown to be activated by a novel antiviral axis involving the *Drosophila* ortholog of the stimulator of interferon genes (STING) protein [83]. This cascade, however, does not seem to be conserved in mosquitoes.

In *Drosophila*, viral infection induces expression of members of the unpaired cytokine family that activate the Jak-Stat pathway to mediate host defense against a specific set of RNA viruses (*Drosophila* C virus, cricket paralysis virus, invertebrate iridescent virus 6) [72, 84-86]. Likewise, persistent infections of fruit flies with nora virus resulted in gene expression profiles that suggest that the Jak-Stat pathway is activated to mediate gut epithelium repair [87]. Also in mosquitoes, the Jak-Stat pathway mediates host defense to viral infection. For example, dengue virus activated the Jak-Stat pathway to induce expression of putative antiviral effectors in *Ae. aegypti* [88] and this pathway was also proposed to mediate antiviral activity against West Nile virus in *Cx. quinquefasciatus* mosquitoes [89, 90]. Finally, in one of the few studies on the role of immune signaling pathways in *An. gambiae*, the Jak-Stat and IMD pathways were shown to suppress O'nyong-nyong virus (ONNV) infection in the midgut [65].

The critical function of NF- κ B pathways in antiviral defense is further illustrated by studies in *Drosophila*, showing that two DNA viruses suppress the Toll and IMD pathways (IIV-6 suppressed both pathway; Kallithea virus suppressed the Toll pathway) [91, 92]. The immune modulatory effect of IIV-6 was further illustrated by

the increased mortality of flies coinfecting with the bacterium *Erwinia carotovora* [91]. In mosquitoes, dengue virus was proposed to actively suppress the Toll pathway in the *Ae. aegypti* Aag2 cell line [93], and ONNV was proposed to inhibit the activity of the Toll, IMD and JAK-STAT pathways in reporter assays in cell culture, which merits further confirmation *in vivo* [65]. Likewise, Semliki Forest virus infection reduced cellular gene expression, perhaps as a mechanism to inhibit immune signaling pathways of the host [94]. Suppression of the IMD pathway was also observed for several DNA viruses of Lepidoptera, which encode homologs of the *Drosophila* cytokine Dieldel that negatively regulates the IMD pathway [84].

Virus-parasite coinfections

To our knowledge, only a single paper directly assessed how virus infection of *Anopheles* mosquitoes affects parasite development [66]. ONNV infection of *An. gambiae* resulted in the upregulation of a gene encoding a fibrinogen-like protein FREP50 and genes involved in the humoral response against *Plasmodium* (LRIM and TEP genes), but also upregulation of negative regulators of the melanization cascade [66]. Mosquitoes were intrathoracically inoculated with ONNV and 48 hours later fed on *P. berghei* containing blood; ONNV infection did not significantly affect the number of live oocysts, but did reduce the number of melanized ookinetes [66]. Whilst data are sparse and interactions should be more comprehensively studied, the study by Waldoock and colleagues provides proof-of-concept for potential interactions between viruses and parasites in *Anopheles* mosquitoes.

Conclusion: can insect-specific viruses affect malaria transmission?

In this review, we explored whether ISV infection of *Anopheles* mosquitoes may affect malaria transmission and describe the immune mechanisms that may mediate this transmission modulation. During its life cycle in the mosquito, the malaria parasite is targeted by different immune responses, some of which are also activated or suppressed by viral infection in dipteran insects (Table 1, Figure 1). It is therefore plausible that persistent ISV infection may prime or activate mosquito immunity to modulate *Plasmodium* development and transmission. Given the broad (but poorly quantified) presence of viral infections in *Anopheles* mosquitoes across continents, the *Anopheles* virome and dynamic changes therein may affect natural malaria transmission dynamics. In addition to potential implications for the force of malaria infection in endemic areas where virus–parasite coinfections in Anophelines occur, ISVs may be explored as possible biocontrol agents against malaria transmission.

We suggest that ISVs may be introduced into natural mosquito populations to interfere with malaria transmission to humans, analogous to the *Wolbachia* program to fight dengue virus. To develop such a strategy, it will be essential to identify an ISV that robustly interferes with *Plasmodium* development or transmission using different *Anopheles* and *Plasmodium* species and strain combinations. Likely, a large reservoir of *Anopheles*-specific ISVs remain to be identified and isolated as candidates for malaria transmission modifying agents. Once a candidate is identified, it will be essential to precisely understand its mechanism of transmission and its prevalence and persistence in *Anopheles* populations. An ideal candidate would be mosquito species specific, efficiently transmitted within the population, and not affect mosquito viability and fecundity. We emphasize, however, that experimental evidence for ISV–parasite interactions is currently lacking and that the biocontrol concept remains to be experimentally explored.

Several other, important knowledge gaps remain to be addressed. First, while the immune response to the malaria parasite is relatively well characterized, only few studies have analyzed the response to virus infection in *Anopheles* mosquitoes [65, 66]. Second, mosquito antiviral immunity has mostly been studied in the context of arbovirus infections, and the response to ISV infection is almost completely unstudied in mosquitoes. Pioneering studies in *Drosophila* indicate that responses to viral infection may be virus specific [86], and also in mosquitoes there seem to be large differences between different virus-host combinations [64, 77]. It therefore remains to be established whether insights from arbovirus infections can be simply extrapolated to ISV infections. Third, while this review focusses on mosquito immunity, it remains possible that there are other mechanisms by which viral infection modulates malaria transmission, for instance by behavioral modifications [95], competition for resources, or inducing mosquito morbidity or mortality. Overall, the tripartite interactions between viruses, parasites, and *Anopheles* mosquitoes are largely unexplored, but hold promise for our understanding of malaria transmission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data were used for the research described in the article.

Outline of the thesis

There might be potential for insect-specific viruses (ISVs) as biological control agents which reduce malaria transmission. The rationale for ISVs as antiparasitic effectors can be exerted from mosquito immune system activation upon viral infection and is extensively discussed before. In *chapter 2*, we give an overview, including a searchable database, about which ISVs are currently discovered within the *Culex*, *Aedes* and *Anopheles* mosquito genera. In *chapter 3*, we conduct a sRNA sequencing study of *Anopheles gambiae* mosquitoes collected from Goden, a rural area located 30 km from Ouagadougou, Burkina Faso, in which we identify a novel partitivirus and possible EVE sequences. In *chapter 4*, we describe the methods needed to successfully infect *Anopheles stephensi* mosquitoes with *Plasmodium falciparum* parasites. These methods are then utilized in *chapter 5*, where we conducted co-infections in *An. stephensi* mosquitoes with ISVs and malaria parasites to test possible effects on sporozoite formation. *Chapter 6* focusses on mosquito immunity after chikungunya (CHIKV) infection. We found that heat shock factor 1 (Hsf1) regulates eight small heat shock proteins (sHsps) which act as an early response against CHIKV infection in *Aedes aegypti* mosquitoes. This cascade seems to be pan-antiviral against not only CHIKV, but also Sindbis and dengue virus as well as the insect-specific Agua Salud alphavirus and O'nyong-nyong arbovirus in *Aedes* and *Anopheles* cells respectively. This data suggests that the Hsf1-sHsps cascade can serve as a promising target for the development of novel intervention strategies against arbovirus transmission by mosquitoes.

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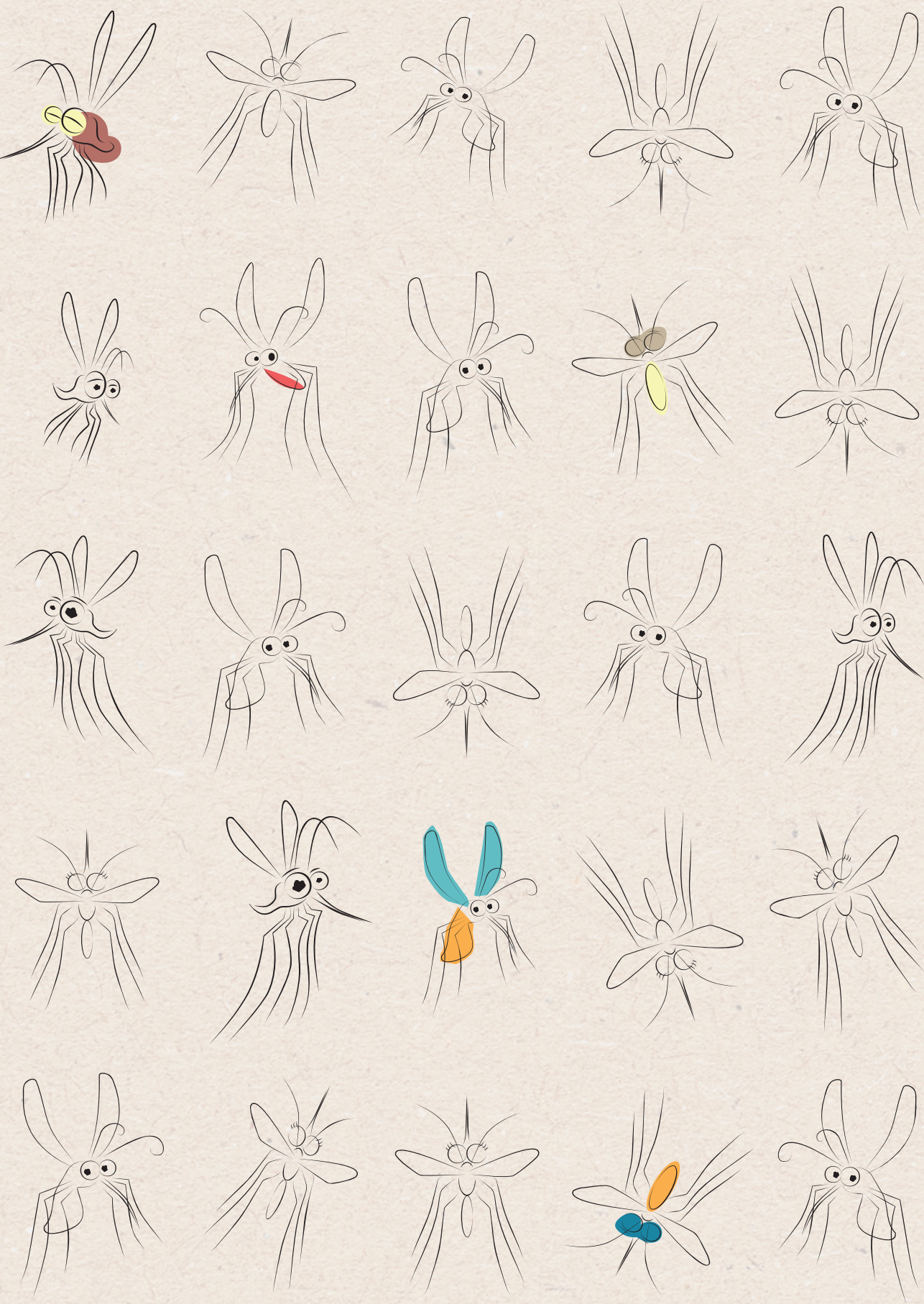
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CHAPTER 2

The composition and global distribution of the virome of mosquitoes - a comprehensive database

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Abstract

Mosquitoes are vectors for emerging and re-emerging infectious viral diseases of humans, livestock and other animals. In addition to these arthropod-borne (arbo) viruses, mosquitoes are host to an array of insect-specific viruses, collectively referred to as the mosquito virome. Mapping the mosquito virome and understanding if and how its composition modulates arbovirus transmission is critical to understand arboviral disease emergence and outbreak dynamics. In recent years, next-generation sequencing as well as PCR and culture-based methods have been extensively used to identify mosquito-associated viruses, providing insights into virus ecology and evolution. Until now, the large amount of mosquito virome data, specifically those acquired by metagenomic sequencing, has not been comprehensively integrated. We have constructed a searchable database of insect-specific viruses associated with vector mosquitoes from 175 studies, published between October 2000 and February 2022. We identify the most frequently detected and widespread viruses of the *Culex*, *Aedes* and *Anopheles* mosquito genera and report their global distribution. In addition, we highlight the challenges of extracting and integrating published virome data and we propose that a standardized reporting format will facilitate data interpretation and re-use by other scientists. We expect our comprehensive database, summarizing mosquito virome data collected over 20 years, to be a useful resource for future studies.

Keywords

Virome, Insect-specific viruses, Mosquito, *Aedes*, *Culex*, *Anopheles*, Metagenomics

Introduction

Hematophagous mosquitoes are vectors for the transmission of arthropod-borne viruses (arboviruses) to humans, livestock and wild animals. Particularly, mosquitoes of the genera *Aedes* and *Culex* transmit epidemic arboviruses, including dengue virus, yellow fever virus, Zika virus and West Nile virus [1-3]. Mosquitoes of the genus *Anopheles* are the main vector for O'nyong-nyong virus as well as for malaria parasites [4]. Besides arboviruses, which have a dual host range alternating between vertebrates and arthropods, mosquitoes carry viruses with an insect-restricted host range (insect-specific viruses, ISVs), as well as viruses that infect microbes such as the bacteria and fungi that colonize the mosquito host [5, 6]. These mosquito-associated viruses are collectively referred to as the mosquito virome.

Virome studies of mosquitoes, and invertebrates in general, have shed light on the vast diversity of viruses on earth [7, 8]. In recent years, next-generation sequencing, PCR-based detection, and virus culture approaches have been extensively used to map the virome across mosquito genera, ecological environments, and geographical locations. These studies expanded the host range of virus families to include arthropods (e.g., in the *Totiviridae* and *Partitiviridae* families [9-11]), introduced new clades within existing viral families or orders (e.g., *Artivirus* in the *Totiviridae* [9] and *Goukovirus*, *Herbevirus*, *Jonvirus* and *Feravirus* in the *Bunyavirales* [12-14]), and necessitated the creation of novel viral families and genera (e.g., *Mesoniviridae* and *Negevirus* [15-18]). Additionally, fundamental insights into virus evolution may be obtained from these studies, as exemplified by the discovery of Nam Dinh virus (or alphamesonivirus 1) in mosquitoes, which led to the establishment of a new family *Mesoniviridae* in the order *Nidovirales*, containing viruses with a genome size intermediate between the small-sized *Arteriviridae* and the large-sized *Coronaviridae* and *Roniviridae* [16].

The mosquito virome has raised significant interest because of its potential impact on the transmission of arboviruses or malarial parasites [19-24]. Correlating spatiotemporal virome data with vector-borne disease incidence may provide insights into the impact of ISVs on pathogen transmission. Moreover, for many ISVs, the host range and the potential to cross the species barrier and infect other (vertebrate or invertebrate) animals remains to be established. Virus infection may impact mosquito physiology and development, which is almost completely uncharacterized thus far but may, directly or indirectly, affect vectorial capacity. Thus, for a One Health perspective on arbovirus transmission, a systematic overview of the prevalence of mosquito-specific viruses is essential. Such an overview may also inform biotechnological

applications of ISVs, such as the development of novel vaccine platforms, or their use as biological agents to prevent arbovirus transmission by mosquitoes [22, 25-27].

Arboviruses are mainly transmitted horizontally between mosquito and vertebrate hosts. In contrast, during adverse conditions such as cold winters or drought, it is hypothesized that arboviruses are vertically transmitted, even if it may be relatively inefficient [28, 29]. ISVs are often assumed to be transmitted vertically from parent to offspring, but direct experimental support for this transmission mode is scarce and both vertical and horizontal transmission routes have been proposed [30, 31]. Virome studies could be used to deduce transmission modes. For example, frequent recovery of an ISV from early life stages such as eggs or larvae could be indicative of vertical transmission, whereas recovery of the same virus from different mosquito species would suggest a horizontal transmission mode via the environment, such as shared food sources.

It is likely that the virome differs between mosquito species, between populations of the same species of mosquitoes, and between individual mosquitoes within populations, which may depend on the transmission mode as well as on viral and host genetics, mosquito ecology, and environmental and climatic conditions. Yet, some ISVs may be present in mosquito populations across the globe or have a broad mosquito host range. For those viruses, it will be particularly relevant to determine their impact on mosquito physiology, development and pathogen transmission.

The large amount of mosquito virome information has thus far been integrated at different levels of analysis. Some studies compared their acquired metagenomic data with sequencing data from other studies [32-35] and several reviews have collated lists of (insect-specific) viruses detected in mosquitoes [5, 25, 36]. However, an exhaustive analysis of mosquito-associated viruses, including their location and associated mosquito hosts, is lacking. In this study, we performed a comprehensive review of 175 mosquito virome studies, published between October 2000 and February 2022, to construct a searchable database of mosquito-associated viruses. We present the most widespread and frequently detected insect-specific viruses within the *Culex*, *Aedes* and *Anopheles* mosquito genera and highlight viruses with a particularly broad mosquito host range. We expect our database to be a useful resource for further study of insect-specific viruses.

Methods

Search strategy

A PubMed search was performed on January 26, 2022, using a combination of title/abstract (Tiab) search terms and Medical Subject Headings (MeSH) terms. The search strategy combined the following terms for (insect-specific) virus discovery with terms for mosquito research:

("Virome"[MeSH Terms] OR "Metagenomics"[MeSH Terms] OR "Insect Viruses"[MeSH Terms] OR "Metatranscriptomic*" [Title/Abstract] OR "Meta transcriptomic*" [Title/Abstract] OR "Metagenom*" [Title/Abstract] OR "Insect Specific Virus*" [Title/Abstract] OR "ISV" [Title/Abstract] OR "Virus Discovery" [Title/Abstract] OR "Virom*" [Title/Abstract] OR "Insect Specific Flavivirus*" [Title/Abstract] OR "Insect Specific Alphavirus*" [Title/Abstract] **AND** ("Culicidae" [MeSH Terms] OR "Culicid*" [Title/Abstract] OR "Aedes" [Title/Abstract] OR "Anophel*" [Title/Abstract] OR "Culex" [Title/Abstract] OR "Mosquit*" [Title/Abstract]).

The search strategy retrieved 743 articles, which were manually screened. All articles written in English and reporting primary data on virus detection or identification in wild-caught mosquitoes were eligible for the analysis. Articles that only detected arboviruses in mosquitoes were excluded, leading to a final selection of 175 articles (references in Supplementary file 1).

Database assembly

Information on mosquito-associated viruses was extracted from the articles at the level of individual samples, containing either a single mosquito or a pool of mosquitoes, to construct a sample-structured database (Supplementary Table S1). Known arboviruses were not included in the table. Each entry in the database constitutes a virus detected in a mosquito sample. Samples tested negative for viruses were not included in the database. For each virus-positive sample, virus taxonomy at the family level, the mosquito species in which the virus was detected, sampling location, blood-feeding status, method for virus detection, material used for sequencing (RNA, DNA or both), the number of mosquitoes in the sample, and the developmental stage (larva, pupa, adult) was extracted from the articles, if this information could be unambiguously deduced. For consistency, *Culex pipiens* was used for studies reporting *Culex pipiens complex* and *Culex pipiens sensu lato* [37, 38]. Likewise, *Ochlerotatus caspius* and *Ochlerotatus scapularis* were denoted as *Aedes caspius* and *Aedes scapularis*, respectively, as both genus names were used in the literature [39]. Virus detection methods were classified into four categories: 1) sequencing, for samples that were directly analyzed by next-generation

sequencing, 2) PCR, for samples in which viruses of a particular virus taxon or species were detected by PCR, 3) culture-sequencing, when mosquito homogenate was cultured on mosquito cell lines, after which viruses were detected by next-generation sequencing, and 4) culture-PCR, when mosquito homogenate was cultured on cell lines and virus was detected by PCR using virus taxon or species specific PCR primers.

Information for the database was extracted from the relevant (supplemental) figures or tables as reported. When viruses were not assigned to the species level, but only the closest viral match was reported, these were included in the database. No thresholds were taken into account for the minimal number of reads and genome coverage required for the accurate detection of viruses, with the exception of the study by Hameed *et al.* [40] (see below). In addition, the authors' assessments were accepted for considering an identified viral sequence novel and giving it a new name. To allow comparison between studies, a column named 'Virus (clean)' was defined, in which strain or isolate names from the 'Virus (reported)' column were removed, virus abbreviations were written out, and consistent spelling was used.

The NCBI Taxonomy database was used as a reference to define unique viruses, as many ISVs are not yet formally classified by the International Committee on Taxonomy of Viruses (ICTV) and therefore absent from the ICTV Master Species List 2021 [41]. For entries without unambiguous reference to a unique virus, 'unknown' was used, except in occasional cases in which the virus name could be deduced from the NCBI taxonomy database using the reported GenBank accession numbers. Virus taxonomy was obtained from the NCBI Taxonomy Database (resourced March through June 2022; [42]) for entries that lack a definition of the virus family in the original article and for viruses with inconsistent taxonomy between articles. In the absence of virus taxonomy at the family level, 'unknown' was used.

Database curation

Initial analysis of the contribution of individual publications to the database indicated that one study dominated the dataset, supplying 4169 of the 8378 (50%) total unique virus entries [40] (Supplemental Figure 1A). This overrepresentation could not be accounted for by the sampling size or sequence depth in this study as only ten pools of mosquitoes were sequenced, which contributed between 121 and 836 virus entries per pool to the database [40]. Instead, for the majority of the reported viruses only a single or few reads were detected and the percentage identity to the viral reference genomes was unreported, providing limited evidence for the presence of these viruses [40]. To prevent a disproportionally large influence of this study on our dataset, a threshold on the minimal number of sequencing reads was

applied and only virus entries supported by ≥ 100 reads were included in the database for this particular study. After this curation, this study supplied 382 of the 4591 (8%) total unique virus entries in our database.

Analyses

Unique virus entries were defined by unique combinations of the columns 'Study', 'Virus family', 'Virus (reported)', 'Location (Specific)' and 'Species' for the analyses of virus families, or the columns 'Study', 'Virus (clean)', 'Location (Specific)' and 'Species' for the analyses at the virus species level. In-house R-scripts were used for data analyses.

Results

We performed a review of the literature on virus identification in wild-caught mosquitoes. Based on a total of 175 publications, we generated a database consisting of 11,261 rows, each entry representing a virus detected in a specific sample (Supplementary Table S1). The number of virus entries in this database is biased towards studies that acquire a large number of samples from the same location, in particular PCR studies that often test multiple mosquito pools sampled at the same site. To account for these biases, we used a transformed database for our analyses, which only included rows with unique combinations of Study - Virus - Mosquito species - Location. We refer to the rows of this database as unique virus entries ($n = 4591$) and use it as a metric for the abundance of viruses and virus families.

Overview of the literature

The number of mosquito virome studies has gradually risen over the years, with two studies published between 2000 and 2008 and a total of 27 studies published in 2021 (Figure 1A). A slight majority of these studies ($n = 70$) used next-generation sequencing-based approaches to characterize the virome, whereas PCR-based approaches ($n = 67$) were frequently used to specifically detect viruses from genera known to contain arboviruses and/or ISVs, such as flaviviruses, alphaviruses, phleboviruses, orthobunyaviruses, densoviruses and rhabdoviruses (Figure 1A). In fewer studies, mosquito homogenate was first cultured on mosquito cell lines, often the RNA interference (RNAi)-deficient *Aedes albopictus* C6/36 cell line, followed by PCR ($n = 21$) or next-generation sequencing ($n = 38$) to detect *in vitro* replicating viruses.

Unique virus entries were not equally distributed across virome studies, with the majority of entries (88.1%) derived from metagenomic sequencing studies and only a small percentage from PCR studies (7.2%) and culturing studies (4.7%). Furthermore,

over 50% of the total number unique virus entries were derived from only 10 out of 175 studies (Supplementary Figure 1A).

Mosquitoes were sampled across the globe (Figure 2B,C) with a relatively even distribution of PCR and sequencing-based methods (Figure 1B). Sampling was, however, not uniform across continents, as China, Brazil, and the USA were the main sources of virome information from Asia, South-America and North-America, respectively (Figure 1C).

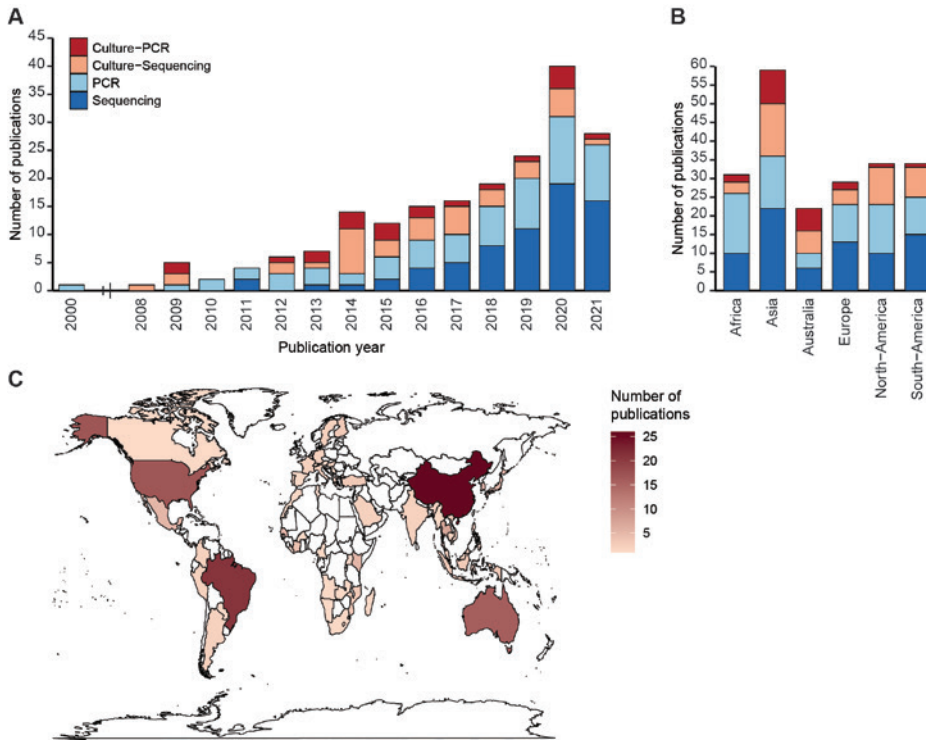


Figure 1. Characteristics of mosquito virome studies. (A-B) Number of mosquito virome publications over time (A), and across continents (B), with fill color indicating the study approach. The sum of the categories within each bar may exceed the actual number of publications as some studies used multiple virus detection methods. (C) Geographic distribution of countries in which mosquitoes were sampled for virome studies.

Mosquito species sampled for virome studies

The 175 studies collectively detected viruses in 128 different mosquito species from 14 mosquito genera (Supplementary Table S2). Mosquitoes from the *Culex*, *Aedes*, and *Anopheles* genera were most frequently found to harbor viruses, likely because these vector mosquitoes are sampled more often for their importance in pathogen

transmission (Figure 2A). *Culex* mosquitoes contributed most unique virus entries in a total of 114 studies, whereas 85 studies detected viruses in *Aedes* mosquitoes, and 34 studies reported viruses in *Anopheles* mosquitoes (Figure 2A, Table 1). As few studies detected viruses in other mosquito genera, we did not further analyze the data from those mosquitoes (Supplementary Table S1).

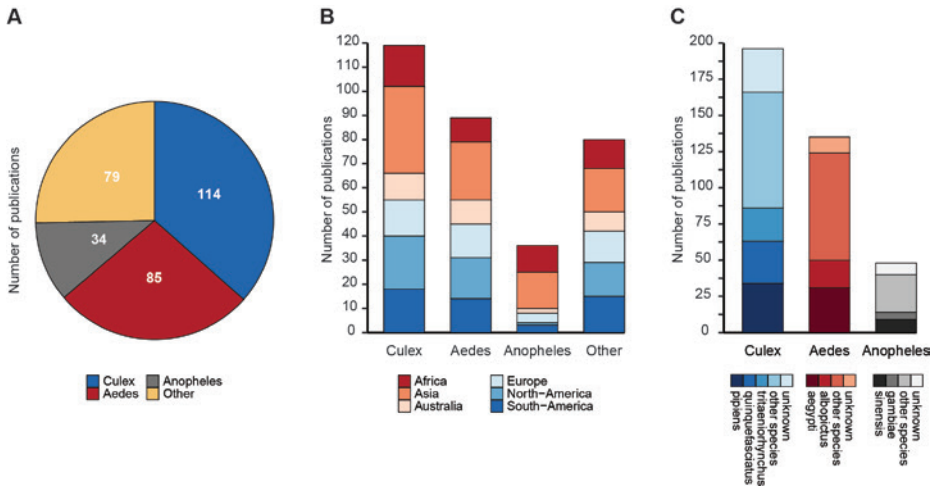


Figure 2. Characteristics of mosquitoes sampled for virome studies. (A) Pie chart indicating the number of publications detecting viruses for each mosquito genus. (B) Number of publications for each mosquito genus with fill color indicating the continent in which mosquitoes were sampled. (C) Number of publications for each mosquito genus, with fill color indicating the most frequently sampled species. Some studies sampled mosquitoes from (A) multiple genera, (B) multiple continents, or (C) multiple species within one mosquito genus, and the sum of publications in each panel therefore exceeds the total number of publications.

Mosquitoes were collected on every continent except Antarctica, with most extensive sampling in Asia and a clear overrepresentation of *Anopheles* sampling in Asia and Africa (Figure 2B). Almost half of the studies reporting viruses in *Culex*, detected these viruses in *Culex pipiens*, *Culex quinquefasciatus* and *Culex tritaeniorhynchus*. For *Aedes* and *Anopheles* mosquitoes, most studies detected virus in *Aedes aegypti* and *Aedes albopictus*, and in *Anopheles sinensis* and *Anopheles gambiae*, respectively (Figure 2C, Supplementary Table S2). Noteworthy, the mosquito species or even the genus was unknown for some virus positive pools, due to studies not defining their species and/or genus [43–45], or due to the use of pooled samples containing multiple species [46–48]. Only 13 studies evaluated the virome in immature developmental such as stages, eggs, larvae, or pupa [32, 49–60].

Table 1. All mosquitoes from the database with the corresponding number of species, continents, countries and studies. Sorted on the amount of studies.

Genus	Species	Continents	Countries	Studies
Culex	33	6	42	114
Aedes	42	6	35	85
Anopheles	23	6	21	34
Mansonia	4	5	11	14
Armigeres	2	1	5	8
Coquillettidia	5	4	5	7
Culiseta	5	3	5	7
Psorophora	5	2	4	6
Ochlerotatus	3	4	5	5
Uranotaenia	1	2	2	3
Sabethes	2	1	1	2
Aedeomyia	1	1	1	1
Heamagogus	1	1	1	1
Wyeomyia	1	1	1	1

General overview of the mosquito virome

In total, viruses from 102 virus families were reported in all mosquito species combined, although the number of unique virus entries was very low for the majority of these families (Supplementary Figure 1B). As expected, virus families known to contain ISVs and/or arboviruses were among the top 10 most frequently observed RNA virus families, including *Flaviviridae*, *Rhabdoviridae*, *Iflaviridae*, *Nodaviridae*, *Mesoniviridae*, *Orthomyxoviridae* and *Totiviridae* (Supplementary Figure 1C, discussed below). In addition, two DNA virus families were found in the top 10 of multiple mosquito genera, *Parvoviridae* and *Genomoviridae*.

Metagenomic surveys have the power to identify viruses of every organism present in the sample. Indeed, bacteriophages were frequently detected, predominantly from the tailed dsDNA bacteriophage families *Siphoviridae* [40, 43, 45, 61-64], *Myoviridae* [34, 40, 45, 62, 64-68], and *Autographiviridae* [40, 65, 67, 69] (Figure S1B). These data underline that metagenomic sequencing can detect viral sequences of bacteria that colonize mosquitoes. Although it remains possible that mosquito physiology is affected by phage infection of bacterial symbionts [70], we have excluded phage families from further analyses.

Positive-stranded RNA viruses

We analyzed the contribution of virus families to the virome of *Culex*, *Aedes* and *Anopheles* mosquitoes, specifically. The *Flaviviridae* family, and specifically the *Flavivirus* genus, contains many mosquito-associated viruses including both arboviruses and ISVs [71, 72]. In our dataset, *Flaviviridae* was the most abundant virus family all mosquito genera (Figure 3). A significant percentage of these entries derived from a few highly abundant viruses, including *Culex* flavivirus (51%) for the *Culex* genus, *Aedes* flavivirus (25%) and Cell fusing agent virus (20%) for the *Aedes* genus, and *Anopheles* flavivirus (23%) for the *Anopheles* genus. These viruses were among the most abundant and widespread in our dataset and have been detected in multiple species within and across mosquito genera (Figure 4). Strikingly, *Culex* flavivirus seems to have a particularly broad host tropism, as it was detected in 12 species of *Culex* mosquitoes as well as three *Aedes* mosquito species and one species of *Anopheles* mosquitoes (Table 2). While flaviviruses are clearly highly prevalent, the family is overrepresented due to the frequent use of PCR studies to detect mosquito-associated flaviviruses, accounting for approximately 60% of *Flaviviridae* unique virus entries (Figure S1C).

The family of *Mesoniviridae* is a recently established taxon of mosquito-infecting positive-sense RNA viruses [17]. The large majority of *Mesoniviridae* virus entries in both *Culex* (74%) and *Aedes* (70%) mosquitoes were derived from alphamesonivirus 1, the founding species of the family that includes several closely related variants, such as Nam Dinh virus, Houston virus and Cavally virus [73, 74]. Alphamesonivirus 1, with most entries from Nam Dinh virus and Houston virus, had a broad global distribution as it was detected in 11 countries across five continents (Table 2,3). Strikingly, alphamesonivirus 1 also had the broadest host range of all viruses in our dataset, as it was detected in 34 mosquito species across five genera.

Nodaviridae and *Iflaviridae* are both well-established families containing insect-associated viruses [75, 76], which were frequently detected in *Culex* and *Aedes* mosquitoes. The majority of these virus entries was derived from only two studies, that sampled in California and China [77, 78]. In China, *Nodaviridae* and *Iflaviridae* sequences, although not classified at the virus species level, were detected in 12 mosquito species [77]. In California, four iflaviruses (*Culex* iflavi-like virus 1-4) and five viruses currently classified as *Nodaviridae* (*Culex* Noda-like virus 1 and *Culex* mosquito virus 1, 2, 3 and 6) were detected in several species of *Culex* mosquitoes, representing 29% of the unique virus entries for *Iflaviridae* and 51% for *Nodaviridae* [78]. *Culex* iflavi-like virus 4 has also been detected in *Culex* mosquitoes in Brazil, China, Belgium and Serbia [65, 79-81] (Table 2).

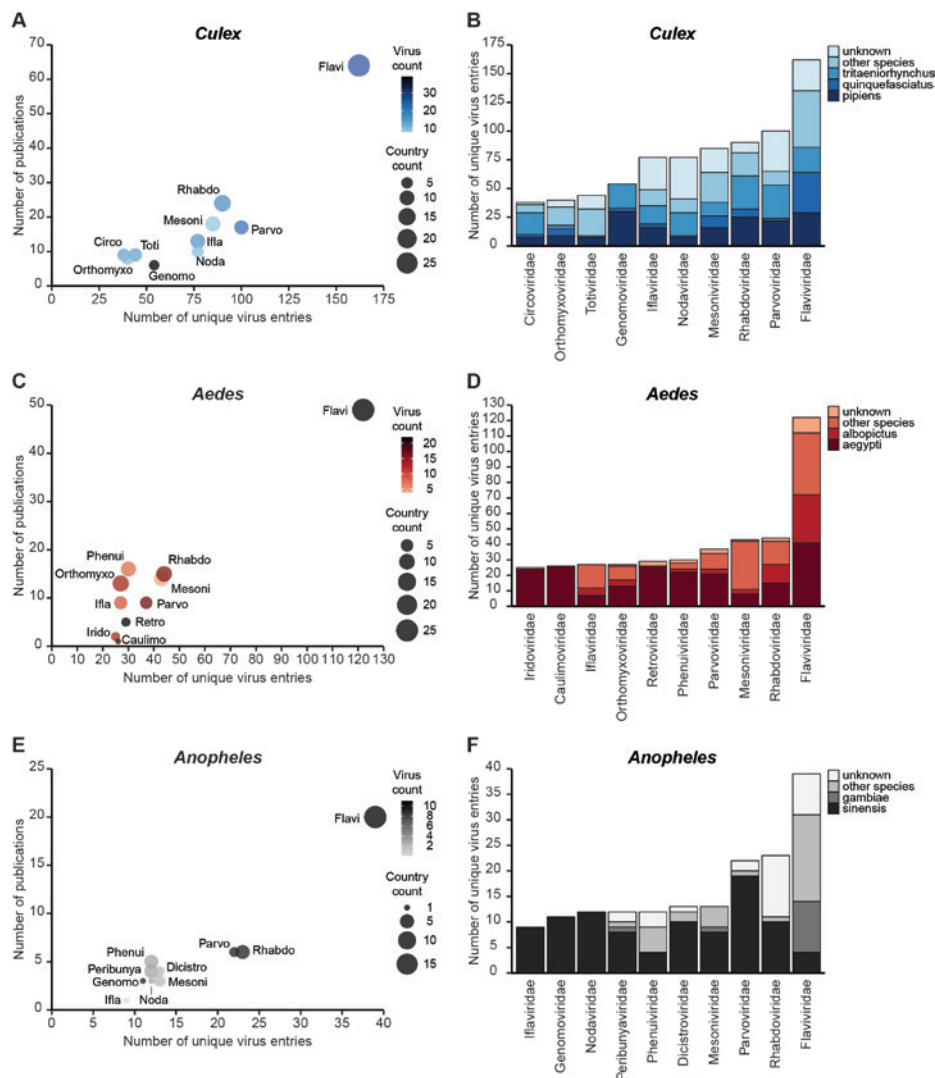


Figure 3. Most frequently reported virus families in mosquitoes. (A, C, E) Top 10 most frequently reported virus families for (A) *Culex*, (C) *Aedes*, and (E) *Anopheles* mosquitoes. The X-axis represents the number of unique virus entries for each family as a measure of virus abundance. The Y-axis indicates the number of studies that reported at least one virus from that family. Fill color indicates the total number of unique viruses detected for each family. Symbol size indicates the total number of countries for each virus family. (B, D, F) Top 10 most frequently reported virus families for (B) *Culex*, (D) *Aedes*, and (F) *Anopheles* mosquitoes with fill color indicating the mosquito species.

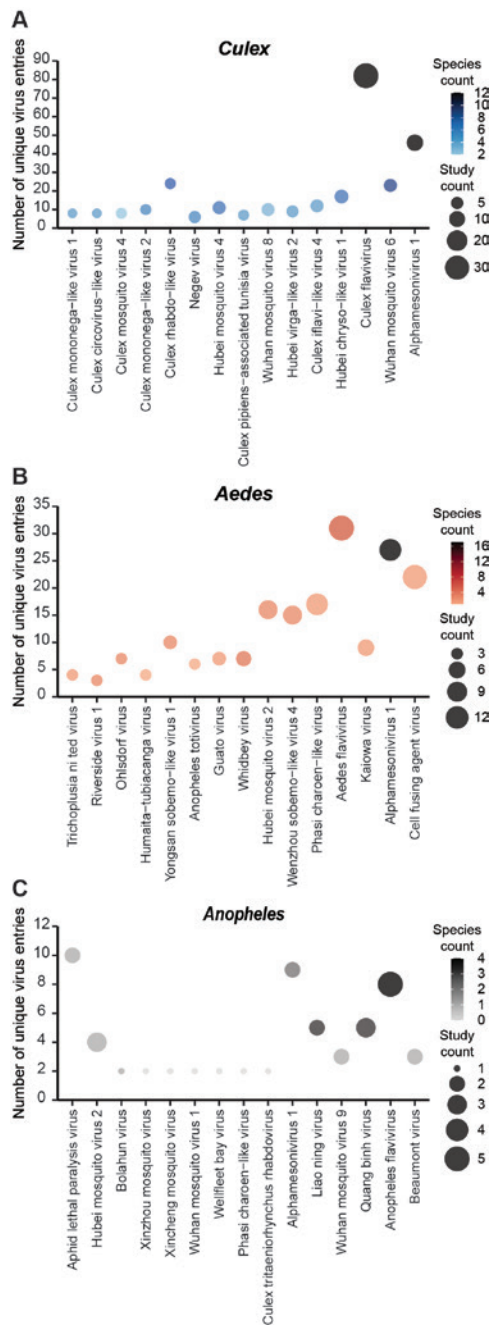


Figure 4. Most widespread mosquito viruses. Top 15 most widespread viruses for (A) *Culex*, (B) *Aedes*, and (C) *Anopheles* mosquitoes. Virus names were ordered according to the number of continents in which the virus was detected. Fill color indicates the number of mosquito species in which the virus has been found within the genus. Symbol size indicates the number of studies in which the virus was found.

Table 2. Most widespread viruses in *Culex* mosquitoes

Virus name	Virus family	Virus genus	Countries within each continent					Studies	Mosquito species	Unique entries
			Africa	Asia	Australia	Europe	North-America			
Alphamesonivirus 1	Mesoniviridae	Alphamesonivirus	-	2	1	3	2	1	11	46
Wuhan mosquito virus 6	Orthomyxoviridae	Quaranjavirus	-	1	1	1	1	1	6	23
Culex flavivirus	Flaviviridae	Flavivirus	2	6	-	-	3	4	33	82
Hubei chryso-like virus 1	Unclassified	Unclassified	-	2	1	2	1	-	7	17
Culex iflavi-like virus 4	Iflaviridae	Unclassified	-	1	-	2	1	1	6	12
Hubei virga-like virus 2	Unclassified	Unclassified	-	1	-	1	2	1	5	9
Wuhan mosquito virus 8	Chuviridae	Culicidavirus	-	1	-	1	1	1	6	10
Culex pipiens-associated tunisia virus	Unclassified	Unclassified	1	1	-	-	1	1	4	7
Hubei mosquito virus 4	Unclassified	Unclassified	-	2	-	-	1	1	6	11
Negev virus	Unclassified	Unclassified	-	1	-	1	-	1	5	6
Culex rhabdo-like virus	Rhabdoviridae	Ohlshavirus	-	1	1	-	1	-	4	24
Culex mononega-like virus 2	Unclassified	Unclassified	-	1	1	1	-	-	4	10
Culex mosquito virus 4	Chuviridae	Culicidavirus	-	1	-	1	1	-	4	8
Culex circovirus-like virus	Circoviridae	Circovirus	-	1	-	-	1	1	3	8

Table 3. Most widespread viruses in *Aedes* mosquitoes

Virus name	Virus family	Virus genus	Countries within each continent					Studies	Mosquito species	Unique entries
			Africa	Asia	Australia	Europe	North-America	South-America		
Cell fusing agent virus	Flaviviridae	Flavivirus	3	4	1	-	3	1	14	2
Alphamesonivirus 1	Mesoniviridae	Alphamesonivirus	-	1	1	1	3	1	11	17
Kaiowa virus	Unclassified	Unclassified	-	2	1	1	1	1	6	2
Aedes flavivirus	Flaviviridae	Flavivirus	2	3	-	2	-	1	15	6
Phasi charoen-like virus	Phenuiviridae	Phasivirus	-	3	1	-	3	1	11	2
Wenzhou sobemo-like virus 4	Unclassified	Unclassified	-	2	-	2	2	1	8	3
Hubei mosquito virus 2	Unclassified	Unclassified	-	2	-	2	1	1	8	3
Whidbey virus	Orthomyxoviridae	Unclassified	-	1	1	3	-	1	5	4
Guato virus	Unclassified	Unclassified	-	1	-	1	1	1	4	2
Anopheles totivirus	Totiviridae	Unclassified	-	1	1	-	1	1	3	1
Yongsan sobemo-like virus 1	Solemoviridae	Sobemovirus	-	2	-	1	-	1	4	3
Humaita-tubiacanga virus	Unclassified	Unclassified	-	1	1	-	2	-	3	1
Ohlsdorf virus	Rhabdoviridae	Ohlstravirus	-	1	-	1	-	1	3	3
Riverside virus 1	Rhabdoviridae	Unclassified	-	1	-	1	-	1	3	3

Table 3. Continued

Virus name	Virus family	Virus genus	Countries within each continent					Studies	Mosquito species	Unique entries
			Africa	Asia	Australia	Europe	North-America	South-America		
Trichoplusia ni ted virus	Metaviridae	Errantivirus	-	1	-	-	1	1	2	4
Hubei toti-like virus 10	Unclassified	Unclassified	-	1	1	1	-	-	2	3
Menghai flavivirus	Flaviviridae	Flavivirus	-	1	-	-	1	1	2	3
Dsrna virus environmental sample	Unclassified	Unclassified	-	-	1	1	1	-	2	3
Croada virus	Unclassified	Unclassified	-	1	-	-	1	1	1	3
Blackford virus	Unclassified	Unclassified	-	1	1	-	1	-	1	3

Negative-stranded RNA viruses

Members of the *Rhabdoviridae* family were among the most frequently observed across mosquito genera in our dataset (Figure 3) and included several of the most widespread viruses, including *Culex* rhabdo-like virus and Merida virus for *Culex* mosquitoes, Ohlsdorf virus and Riverside virus 1 for *Aedes* mosquitoes, and Beaumont virus and Wuhan mosquito virus 9 for *Anopheles* mosquitoes, which were each detected in at least three countries (Table 2, 3, 4). Notably, Merida virus has been found in four continents and in mosquito species of the genera *Culex*, *Aedes* and *Heamagogus* [32, 33, 40, 66, 79, 82].

The *Xinmoviridae* family was established in 2017 to encompass the free-floating genus *Anphevirus* in the *Mononegavirales* order [83]. Anpheviruses were detected in multiple mosquito species, including Xincheng mosquito virus and Bolahun virus in *Anopheles* mosquitoes and *Aedes aegypti* anphevirus, *Aedes albopictus* anphevirus and *Aedes anphevirus* in *Aedes* mosquitoes [66, 79, 81, 84-86]. Notably, the contribution of *Xinmoviridae* to the mosquito virome may be underestimated due to the recent establishment of this family.

Several segmented viruses of the *Orthomyxoviridae* and *Phenuiviridae* were among the most widespread in *Culex* and *Aedes* mosquitoes (Figure 4A,B). For the *Orthomyxoviridae*, these included Wuhan mosquito virus 6 for *Culex* mosquitoes and Whidbey virus for *Aedes* mosquitoes (Table 2,3). In particular, Wuhan mosquito virus 6 showed a near-global distribution and broad mosquito host range, as it was detected in eight countries across all continents except Antarctica, and in 12 mosquito species across four genera (Supplementary file 1). *Phenuiviridae* was among the most frequently detected virus families in *Aedes* mosquitoes due to the high prevalence of Phasi Charoen-like virus, which contributed 60% of the entries of this family. Phasi Charoen-like virus is one of the most widespread viruses in *Aedes aegypti* specifically (Table 3), although it was also detected in *Aedes albopictus* [32], *Culex quinquefasciatus* [85], *Haemagogus janthinomys* [82] and *Anopheles* mosquitoes [87].

Table 4. Most widespread viruses in *Anopheles* mosquitoes

Virus name	Virus family	Virus genus	Countries within each continent					Studies	Mosquito species	Unique entries
			Africa	Asia	Australia	Europe	North-America			
Beaumont virus	Rhabdoviridae	Unclassified	1	1	1	-	-	2	1	3
Anopheles flavivirus	Flaviviridae	Unclassified	5	-	-	1	-	5	4	8
Quang binh virus	Flaviviridae	Unclassified	2	1	-	-	-	3	3	5
Wuhan mosquito virus 9	Rhabdoviridae	Unclassified	1	2	-	-	-	2	1	3
Liao ning virus	Reoviridae	Seadornavirus	-	1	1	-	-	2	3	5
Alphamesonivirus 1	Mesoniviridae	Alphamesonivirus	-	1	1	-	-	2	2	9
Culex tritaeniorhynchus rhabdovirus	Rhabdoviridae	Merhavirus	1	1	-	-	-	1	n.d.*	2
Phasi charoen-like virus	Phenuiviridae	Phasivirus	1	1	-	-	-	1	n.d.*	2
Wellfleet bay virus	Orthomyxoviridae	Quaranjavirus	1	1	-	-	-	1	n.d.*	2
Wuhan mosquito virus 1	Phasmaviridae	Orthophasmavirus	1	1	-	-	-	1	n.d.*	2
Xincheng mosquito virus	Xinmoviridae	Anphevirus	1	1	-	-	-	1	n.d.*	2
Xinzhou mosquito virus	Peribunyaviridae	Unclassified	1	1	-	-	-	1	n.d.*	2
Chaq virus-like 2	Unclassified	Unclassified	3	-	-	-	-	1	1	3
Partitiviridae-like 2	Partitiviridae	Unclassified	3	-	-	-	-	1	1	3

Table 4. Continued

Virus name	Virus family	Virus genus	Countries within each continent					Studies	Mosquito species	Unique entries
			Africa	Asia	Australia	Europe	North-America	South-America		
ssRNA virus-like 5	Unclassified	Unclassified	2	-	-	-	-	-	1	3
Bolahun virus	Xinmoviridae	Anphevirus	2	-	-	-	-	-	1	2
ssRNA virus-like 6	Unclassified	Unclassified	2	-	-	-	-	-	1	2
Chaq virus-like 3	Unclassified	Unclassified	2	-	-	-	-	-	1	2
Partitiviridae-like 3	Partitiviridae	Unclassified	2	-	-	-	-	-	1	2
Hubei mosquito virus 2	Unclassified	Unclassified	-	1	-	-	-	-	3	4

* n.d., *Anopheles* mosquitoes not defined at the species level* n.d., *Anopheles* mosquitoes not defined at the species level

Double-stranded RNA viruses

The *Artivirus* genus in the family *Totiviridae* is comprised of double-stranded RNA viruses of arthropods, including mosquitoes [9, 88]. *Totiviridae* entries in our database corresponded to multiple totiviruses, with limited cross-detection between studies. Notably, the most frequently detected totivirus was *Anopheles totivirus* which, after its initial detection in *Anopheles gambiae* in Liberia [86], was found in *Aedes aegypti* in several countries across multiple continents [66, 67, 69] (Table 3).

DNA virus families

The *Parvoviridae* family of single-stranded DNA viruses is the most abundant DNA virus family in all three mosquito genera (Figure 3). The family contains densoviruses, which have been studied as a potential biological control agent of insects and mosquitoes specifically [89]. A large proportion of the *Parvoviridae* entries for all three mosquito genera was derived from a single study [77], which detected parvovirus sequences in several *Culex*, *Aedes* and *Anopheles* species across China. An additional 38% of the *Parvoviridae* entries for *Culex* mosquitoes corresponded to *Culex* densovirus, which was detected in *Culex pipiens* and mixed pools of *Culex* mosquitoes across California [78].

Two families of circular Rep-encoding single-stranded DNA viruses (also referred to as CRESS-DNA viruses; [90]), *Genomoviridae* and *Circoviridae*, were frequently detected (Figure 3; Figure 4A). The high abundance of *Genomoviridae* in our dataset was mostly due to three metagenomic sequencing studies, each detecting sequences mapping to multiple genomoviruses [40, 61, 67]. For the *Circoviridae*, *Culex* circovirus-like virus was detected in three studies over three continents in species of all three genera [40, 78, 81].

For both *Genomoviridae* and *Circoviridae*, novel species have mostly been detected through metagenomic sequencing [91, 92]. As active replication of these viruses has not been described in the animals sampled for sequencing, it is possible that these viruses are associated with food sources or pathogens of the host, precluding conclusions on the host range of these virus families [91, 92]. However, for viruses from the *Circoviridae*, and specifically the *Cyclovirus* genus, arthropods (and mosquitoes specifically) have been suggested to be the primary host [67, 93]. Moreover, Sclerotinia sclerotiorumhypovirulence-associated DNA virus 1, the founding species of the *Genomoviridae* viral family, was found to infect the mycophagous mosquito species *Lycoriella ingenua* under experimental conditions [94]. These studies suggest that vector mosquitoes could be a part of the host range of genomoviruses and circoviruses.

Unclassified viruses

The majority of virus entries in our database are unclassified at the family level (Figure S1B). This large group included 114 unique virus entries from the genus *Negevirus* [18], a taxon of insect-specific, non-segmented, enveloped, positive-sense RNA viruses that has not been classified yet at the family level. Negeviruses are among the most abundant virus taxa in *Culex* (53 unique virus entries) and *Aedes* (32 unique virus entries) mosquitoes. Negeviruses have been reported in multiple *Culex* and *Aedes* species and across at least four continents. Among these, the most abundant virus was the eponymous Negev virus, which was detected in *Aedes aegypti* [81] and several *Culex* species across three continents [33, 65, 95, 96] (Figure 4A).

Most frequently detected viruses per mosquito genus

We collated lists of the top 20 most frequently detected viruses for each mosquito genus according to the number of continents and countries in which they were detected (Table 2, 3, 4, Figure 4). For *Aedes* and *Culex*, the broad global distribution of these viruses was well supported, being detected in multiple countries in two to five continents in at least three independent studies, lending support to the validity of these observations. Surprisingly, all or nearly all top 20 viruses for *Culex* and *Aedes* respectively, were detected in multiple mosquito species, suggesting that vertical transmission is not the sole transmission route for these viruses. In line with the more limited sampling of *Anopheles* mosquitoes (Figure 2), the top 20 *Anopheles* viruses were detected less frequently (between 2 and 9 unique virus entries) and at fewer places across the globe (Figure 4C; Table 4).

A large number of unclassified viruses were among the top 20 (ten for *Culex*, nine for *Aedes*, and five for *Anopheles*; Table 2-4). Among these, some have a particularly broad global distribution, having been detected in at least four continents. These include Hubei chryso-like virus 1, Hubei virga-like virus 2 and *Culex pipiens*-associated Tunisia virus for *Culex* mosquitoes, and Kaiowa virus, Whenzhou Sobemo-like virus and Hubei mosquito virus 2 for *Aedes* mosquitoes (Table 2,3). Notably, the detection of Kaiowa virus in *Aedes aegypti* metagenomic studies was proposed to be due to the presence of endogenous viral elements in mosquito genomes instead of replicating virus [71]. Metagenomic studies have also reported sequences with homology to Kaiowa virus in samples from *Aedes albopictus*, *Culex quinquefasciatus* and *Heamagogus janthinomys* [32, 82, 85, 97] and the origin of these sequences merits further investigation.

Discussion

Growing scientific interest and increasing accessibility to deep-sequencing technology has led to a large body of literature on the mosquito virome. We have collated information from 175 research articles from the last 22 years to construct a comprehensive and searchable database of mosquito-associated viruses, along with the locations and hosts in which they have been detected. We found that RNA viruses from the families *Flaviviridae* and *Rhabdoviridae* are widespread in *Culex*, *Aedes* and *Anopheles* mosquitoes globally. We collated lists of the top 20 viruses with the widest global distribution for each of these mosquito genera and found that most of these viruses were detected in multiple mosquito species within, and sometimes across mosquito genera. The prevalence and overall stability of these viruses within mosquito populations and the transmission routes enabling them to persist and spread merits further investigation.

We collated virome data as reported, accepting the authors' assessment for assigning viral sequences to established virus species or taxons or for considering a virus novel. A limitation of this approach is that different thresholds for genome coverage, number of virus mapping reads or contigs, and nucleotide or amino acid sequence identity scores were used for virus identification between studies. More concerningly, some studies did not unambiguously report the criteria used for virus identification, which makes side-by-side comparisons of studies difficult. Consequently, the database contains some low-confidence virus entries due to insufficiently stringent thresholds for virus identification or misclassification of virus sequences. Due to these caveats and differences in sampling intensities, our database cannot be used to accurately infer the absence of a virus in specific mosquito species. Another limitation of our study is that we may have inevitably missed relevant articles that did not match our search terms, despite our best efforts to use a comprehensive literature search strategy. Despite these limitations, the most widespread and abundant viruses in our dataset have been found in multiple independent studies. As such, the collated top 20 most widespread viruses can be considered high-confidence constituents of the mosquito virome, especially for *Aedes* and *Culex* that have been sampled most extensively.

During our study, we noticed that virome data are often impractically reported for interpretation and re-use by other scientists, due to unreported critical variables or an impractical format to present results (e.g., in heat-maps) without accompanying presentation in a reusable data format. We propose that reporting can be improved by the standard inclusion of a supplementary table containing per virus positive

sample, i) the viruses identified along with accession numbers, nucleotide and amino acid identity scores, genome coverage, numbers of reads/contigs mapping to the viral genome, ii) information on the sample, such as pool identifier, number of mosquitoes per sample, mosquito species, sex, and life stage, iii) the date and location of sampling, along with geographic coordinates and type of habitat, and iv) sequencing information, including the sequencing platform, method for library preparation, sequencing depth per library, and accession number of the repository in which the raw sequence data have been deposited.

Next-generation sequencing is a relatively unbiased approach that successfully detects both RNA and DNA viruses in mosquitoes. However, the detection of sequences of well-known mammalian viruses of the *Retroviridae* [40, 65, 66, 82] (e.g., murine leukemia virus), *Herpesviridae* [40, 45, 65] (e.g., herpes simplex virus) and *Hepadnaviridae* (e.g., hepatitis B virus) [61] indicates that some datasets contain considerable amount of noise. The origin of these sequences is unclear but may be due to insufficiently stringent thresholds for virus identification, to biological contaminants such as sequences derived from blood meals, or to experimental contamination during library preparation and sequencing. Alternatively, although no insect viruses have currently been described in the family *Retroviridae*, it remains possible that these sequences derive from unidentified mosquito retroviruses or from retroelements in the mosquito genome.

Detection of viral sequences does not provide direct support of active replication in the mosquito host and, indeed, bacteriophage sequences were frequently detected in mosquito virome studies [34, 40, 43, 45, 65, 67, 69]. Isolation of a virus in mosquito cell culture would provide strong support for active virus replication in the mosquito host [98, 99]. Alternatively, small RNA-sequencing approaches may be used to distinguish sequences of actively replicating viruses from contaminating sequences. Viral double-stranded RNA produced during replication of both DNA and RNA viruses are processed into 21-nt small interfering RNAs [100] that can be readily distinguished in small RNA size profiles. Indeed, some researchers have used small RNA sequencing as an alternative or complement to conventional mRNA sequencing for virome studies in insects [101-104].

Small RNAs may also help to distinguish replicating viruses from another source of viral sequences, EVEs. The *Aedes aegypti* and *Aedes albopictus* genomes contain a large number of non-retroviral EVEs [105-107], transcripts of which may be detected in RNA-seq experiments. Next-generation sequencing studies therefore require careful analysis to differentiate between EVE-derived sequences and virus-derived sequences.

The removal of contigs mapping to mosquito genomes may be impossible for species lacking reference genomes and, even for species with high-quality reference genomes, this may be insufficient as the EVE repertoire differs between mosquito populations [108]. Small RNA sequencing may help to distinguish EVE-derived sequences from sequences of replicating viruses as EVEs may primarily give rise to PIWI-interacting RNAs (piRNAs) with a typical size of 25-30 nt and strong strand biases. These can be readily distinguished from replication-dependent siRNAs of 21 nt, which are usually derived from both positive and negative-sense viral RNAs [102, 108].

The composition of the mosquito virome is likely shaped by the environment, virus transmission modes, and restrictive factors in specific mosquito species or genera. Moreover, changing biotic and abiotic factors associated with global warming and increasing globalization may further affect the mosquito virome. Our database is an up-to-date, comprehensive overview of primary literature on mosquito-associated viruses from the last 22 years. As such, our study forms a solid foundation to study inter- and intra-species pathogen transmission from a One Health perspective. A future challenge will be to understand how virome dynamics affect mosquito-borne disease outbreaks.

Authors' contribution

JPM, MS, TM, PM and RPR contributed to the conceptualization of the study. MS developed the literature search strategy in consultation with data stewards of Radboud University Medical Library. JPM, MS, and TM performed the investigation and data curation, including literature screening, data extraction, and construction of the database. JPM was responsible for visualization. MS and TM wrote the original draft of the manuscript. All authors contributed to review and editing and approved the final version of the manuscript. Shared first authorship is in alphabetical order.

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Declaration of competing interest

None.

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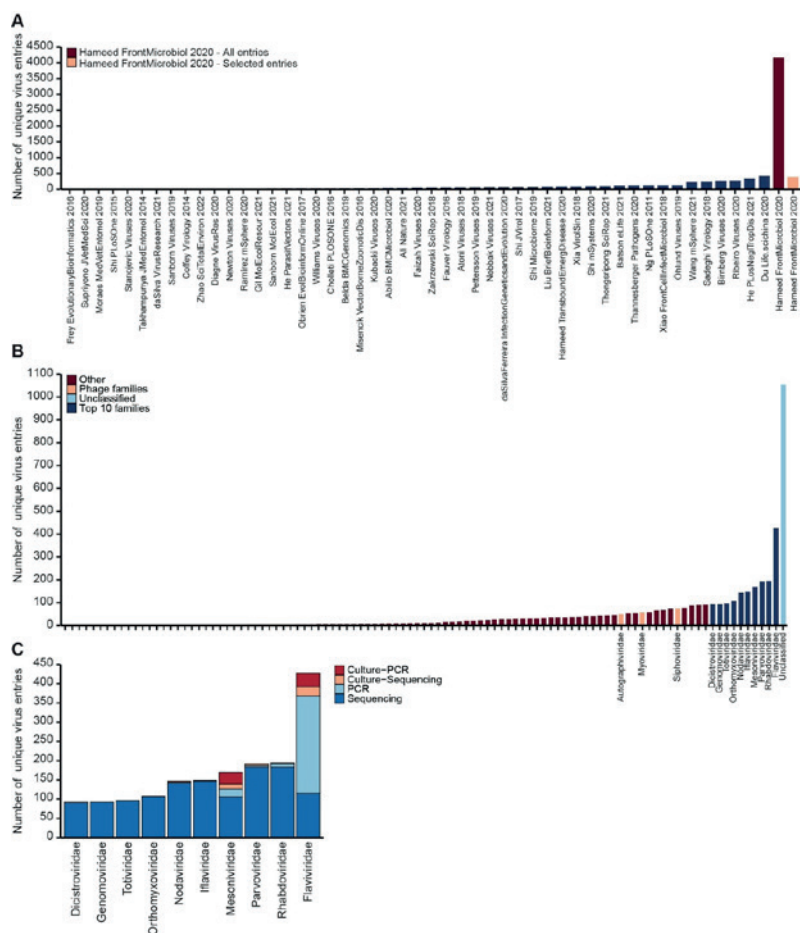
Supplementary Materials

Supplementary file and tables can be downloaded via
<https://www.sciencedirect.com/science/article/pii/S2352771423000101#ecoo10>

Supplementary file 1. Final selection of 175 articles used for the analyses and their references.

Supplementary Table S1. Sample-structured database of viruses identified in mosquito virome studies.

Supplementary Table S2. Virus positive mosquito species, with the corresponding number of continents, countries and studies in which they are sampled.



Supplementary Figure S1. Unique virus entries in mosquito virome studies. (A) Number of unique virus entries reported for the top 50 studies that contributed most entries in our database. Unique virus entries for Hameed et al. (2020) before (dark red) and after (salmon) data curation are indicated. (B) Number of unique virus entries corresponding to the individual virus families for all mosquito species in our database. Bacteriophage families are indicated with salmon fill color; viruses not classified at the family level in light blue. (C) Number of unique virus entries for the top 10 most frequently detected families, according to detection method.



CHAPTER 3

Small RNA sequencing detects high prevalence of partitivirus and endogenous flavivirus sequences in *Anopheles* mosquitoes

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Abstract

Mosquitoes harbor a wide diversity of viruses, many of which are currently uncharacterized. This is particularly true for malaria-transmitting *Anopheles* mosquitoes. We used small RNA sequencing to characterize the virome of a total of 456 female *Anopheles gambiae* mosquitoes collected from Goden, Burkina Faso. The most frequently detected virus was a partitivirus, putatively named Goden partiti-like virus, a multipartite virus comprising three segments, encoding a RNA dependent RNA polymerase, a capsid protein, and a Chaq-like protein of unknown function. The virus was found in 10 out of the 38 mosquito pools tested, suggesting high prevalence. Additionally, sequences matching the *Aedes aegypti* To flavivirus-like virus were found. However, the PIWI-interacting (piRNA)-like signature of mostly 28 nucleotide reads suggests that these sequences derive from an endogenous viral element integrated in the mosquito genome. A subset of 'dark matter' contigs remains unclassified, although their enrichment for small interfering RNA (siRNA)-sized reads of 21 nucleotides suggests that they may represent novel viruses. This study expands the known diversity of mosquito-associated viruses in West Africa and identifies a highly prevalent partitivirus in the sampled population. Moreover, our study confirms the utility of small RNA sequencing to detect both active viral infections and integrated viral elements.

Introduction

Mosquitoes are vectors for various human-pathogenic arthropod-borne (arbo-) viruses, such as chikungunya, Zika, West Nile, O'nyong-nyong and dengue [1-3]. Beyond these, mosquitoes are also hosts for a wide range of insect- and mosquito-specific viruses that are not transmitted to vertebrate animals [4, 5], but may bear relevance for the infected mosquitoes or influence the transmission of vector-borne pathogens. Recent advances and cost reductions in high-throughput sequencing have greatly accelerated the discovery of a large number of previously unknown mosquito viruses [6], most of which are predicted to only replicate within their insect hosts. While these viruses may not have direct impact on public health, they are of significant interest.

Charting the virome in diverse insect hosts provides important insights into virus diversity and evolution [7-9]. Some newly discovered insect viruses belong to arbovirus-related families and may serve as sources of emerging arboviruses, offering insights into traits needed for vertebrate transmission [4, 10]. Moreover, insect viruses may have direct and indirect effects on the transmission of vector-borne diseases. For example, several insect viruses have been shown to affect arbovirus replication and vector competence, and insect viruses may thus be deployed in disease control [11-18]. Moreover, insect viruses may affect the lifespan, fertility and fecundity of mosquito vectors. While not yet studied in detail, such effects may affect the total mosquito population size and hence vectorial capacity for vector-borne diseases.

High-throughput sequencing methods, such as RNA-sequencing, have become widely adopted to characterize the mosquito metagenome [19, 20]. However, despite the clear successes in characterizing the mosquito virosphere, total RNA sequencing has several limitations. First, virus discovery using RNA-sequencing primarily depends on sequence similarity searches onto already existing databases, which inherently biases virus discovery towards relatives of known viruses. As a result, highly divergent viral sequences may go unrecognized, contributing to what is often referred to as 'viral dark material' [21]. Moreover, RNA-sequencing reads do not necessarily originate from active replicating viruses, but instead may be derived from viral sequences integrated into host genomes, called endogenous viral elements (EVEs) [22], and find their origin in the microbiota colonizing the mosquito [23], or environmental contaminants. Finally, if viral sequences are not enriched before sequence library preparation, the vast majority of sequencing reads will likely be of non-viral origin, reducing the sensitivity and efficiency of virome characterization [24]. Small RNA sequencing has been proposed to address some of these limitations, as it taps into the antiviral RNA interference (RNAi) machinery that processes (replicating) viral RNA into small RNAs [24-26].

Most animals possess three distinct RNA silencing pathways, the small interfering RNA (siRNA), PIWI-interacting RNA (piRNA) and microRNA pathways, each defined by distinct mechanisms for small RNA biogenesis, resulting in specific size distributions and nucleotide biases. Of these, the siRNA and piRNA pathways are most relevant for viral detection as they process viral RNAs into small RNAs, whereas virus-derived microRNAs seem to be more rare and primarily associated with DNA viruses [27-29]. In the siRNA pathway of insects, Dicer-2 produces 21-nt siRNAs from viral double-stranded RNA (dsRNA), derived from replication intermediates of RNA viruses, RNA structures, the genomes of dsRNA viruses [30], or dsRNA produced by overlapping convergent transcription [31-33]. Processing of replication intermediates of RNA viruses result in both positive and negative-sense siRNAs, well documented during arbovirus and insect-specific virus infections of mosquitoes [34-39], and can thus be used as a marker of active RNA replication [24-26, 40]. The piRNA pathway of *Aedes* and *Culex* mosquitoes produces viral piRNAs from select viruses [41-44], in a biogenesis mechanism called ping-pong amplification. This mechanism depends on the PIWI proteins Piwi5 and Ago3 and several accessory proteins [45-48] and processes single-stranded RNA precursors into piRNAs of 24-29 nt [41-44].

While the viromes of *Aedes* and *Culex* mosquitoes have been extensively studied, the virome of *Anopheles* mosquitoes remains less well characterized [49, 50]. In this study, we used small RNA sequencing to identify actively replicating viruses in *Anopheles* mosquitoes from Goden, a rural area located 40 km to the East of Ouagadougou, the capital of Burkina Faso. We identified a putative new partitivirus, named Goden partiti-like virus, at high prevalence. In addition, we identified a flavivirus-derived sequence, likely derived from an EVE, and genome coverage for a virgavirus, although the small profile lacked the typical siRNA size expected of replicating viruses. Finally, for several contigs with a clear 21-nt peak, we failed to obtain a similarity hit in viral databases, suggestive of infection with an as-yet unknown, diverse virus.

Results

Mosquito collection

We constructed small RNA libraries from *An. Gambiae* mosquitoes from Goden (12°25' N, 1° 21' W), a rural area located 40 km East of Ouagadougou, the capital of Burkina Faso (Fig1). In this village, malaria is endemic and the infection peak occurs during the rainy season from August to October. The main vectors are *Anopheles gambiae* s.l. (consisting of *An. gambiae* s.s, *Anopheles coluzzii* and *Anopheles arabiensis*) and *Anopheles funestus*. *Plasmodium falciparum* is the predominant malaria parasite, accounting for

more than 95% of infections in children under five [51]. A total of 456 adult, resting, female *Anopheles gambiae* s.l. mosquitoes were caught around households using a prokopack aspirator [52] and individually stored at -80°C . Mosquitoes were then randomly placed into 96 well plates and later pooled into 38 groups of 12 mosquitoes to make small RNA sequencing libraries.

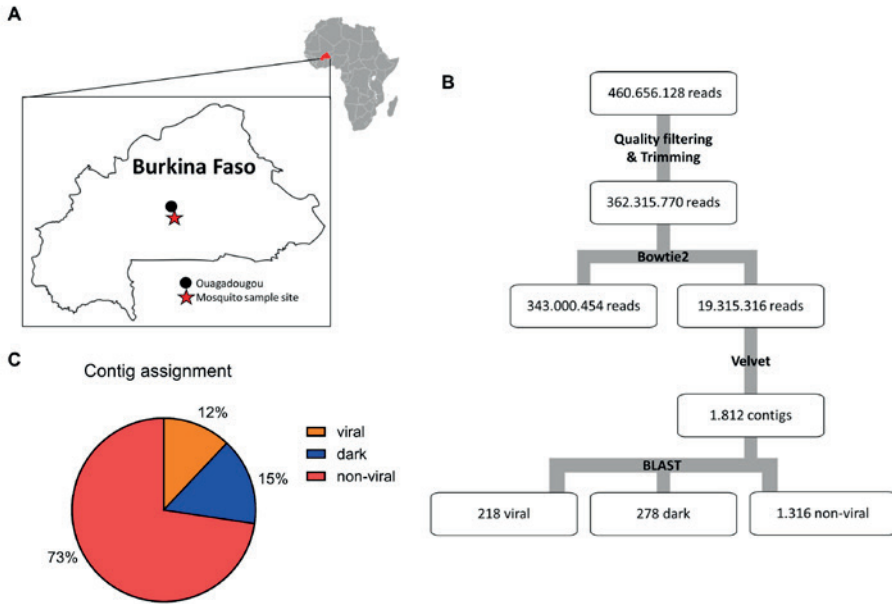


Figure 1. *Anopheles* sample collection and analyses. (A) Geographic map showing the sample location where the *Anopheles* mosquitoes were gathered. (B) Schematic overview of small RNA analysis pipeline showing the total number of reads at each analysis step. (C) Contig assignment into viral, dark and non-viral for all contigs combined.

Goden *Anopheles* virome

After small RNA sequencing, the libraries were processed by adapter trimming, quality checking and mapping to the *An. gambiae* (AGAMP3) genome. Reads mapping to the *An. gambiae* reference, accounting for 85.0–97.2% of the total reads, were removed. Of the remaining reads, contigs were assembled resulting in 1812 contigs larger than 75 nucleotides. BLAST searches for the contigs identified 12.0% of the total contigs as viral, having the highest similarity with a known virus. 72.6% of the contigs have the highest similarity to non-viral sequences, and the remaining contigs lacking significant similarities in the database searches (15.3%) were annotated as ‘dark’ material (Fig1, Sup1, Sup2). Several contigs in the nonviral category showed significant similarity with the *Anopheles* genome. These sequences most likely derive from the host genome but were not removed during the AGAMP3 mapping

step, probably due to differences between the genomes of the sampled mosquitoes (*Anopheles* species complex) and the reference genome, which is derived from an extinct lab colony, a mixture of two species, *Anopheles gambiae sensu stricto* and *Anopheles coluzzii* [53, 54]. Other nonviral sequences are derived from bacteria and fungi and could be part of the mosquito microbiome. However, as households are non-sterile, it is also possible that these are environmental contaminations in the samples. Some contigs had their closest hit with mammals like human and cattle, suggestive of a signal from a blood meal on these animals. The number of viral contigs, a total of 218, varied between 0 to 28 per sample. Within the 218 viral derived contigs, we found 34 different viral hits (Table1, Sup3) of which 13 were identified via nucleotide-to-nucleotide BLAST (BLASTn) and 20 through translated nucleotide-to-protein (BLASTx) search.

The identified viruses could be classified into 10 different viral families ($n = 129$ contigs), the remaining 89 contigs were from unclassified viruses. Several samples contained different contigs matching to various highly similar viruses, for example partitivirus-like 2 and 3 in sample 1E, 1F, 2H, 3A, 3D, 4F and 4H, or *Anopheles* flavivirus-like 1 and *Anopheles* flavivirus variant 1 in sample 3F. These contigs likely represent a single virus of which different parts of the genome have highest BLAST similarity to different viruses. Contigs covered over 20% of the accession of Partitivirus-like 2 and 3, Chaq virus-like 2 and 3, Orestiada partiti-like virus, *Aedes aegypti* To flavivirus-like, Hubei Virga-like virus 21 and Nefer virus, and will be further discussed. Subsequently, we will examine the contigs annotated as dark material in order to characterize their potential origin.

Table 1. Virallhits. Classification of viral Blast hits identified in *Anopheles* mosquitoes from Goden, Burkina Faso, sorted by highest accession coverage of each hit. The total coverage of each found accession is shown as the length which is covered by the contigs over the total accession length. The different viral hits represent the contigs' closest viral match within the database.

Blast alignment hit	Viral family	Description	Search strategy	Accessions	Samples	Accession coverage
Partitivirus-like 2	Partitiviridae	Partitivirus-like 2 RdRp gene, partial cds / RdRp [Partitivirus-like 2]	Blastn / Blastx	KX148576.1 / AOR51389.1	1E, 1F, 1G, 2F, 2H, 3A, 3D, 4F, 4H	97,7%
Chaq virus-like 2	Unclassified	Chaq virus-like 2 ORF1 gene, complete cds / ORF1 [Chaq virus-like 2]	Blastn / Blastx	KX148555.1 / AOR51385.1	1A, 1E, 1F, 1G, 2F, 2H, 3A, 3D, 4F, 4H	95,1%
Chaq virus-like 3	Unclassified	Chaq virus-like 3 ORF1 gene, complete cds / ORF1 [Chaq virus-like 3]	Blastn / Blastx	KX148556.1 / AOR51386.1	1A, 1E, 1F, 1G, 2F, 2H, 3A, 3D, 4F, 4H	93,8%
Partitivirus-like 3	Partitiviridae	Partitivirus-like 3 RdRp gene, complete cds / RdRp [Partitivirus-like 3]	Blastn / Blastx	KX148577.1 / AOR51390.1	1E, 1F, 2F, 2H, 3A, 3D, 4F, 4H	86,9%
Orestia da partiti-like virus	Partitiviridae	putative capsid protein [Orestia da partiti-like virus]	Blastx	QRD99858.1	1E, 1F, 1G, 3A, 3D, 4F	74,4%
Aedes aegypti To flavivirus-like	Flaviviridae	Aedes aegypti To flavivirus-like isolate AaTFLV/BR_TO nonstructural protein 3 gene, complete cds / nonstructural protein 3 [Aedes aegypti To flavivirus-like]	Blastn / Blastx	MT913594.1 / QPF16706.1	1D, 1E, 1F, 1G, 1H	39,6%
Hubei virga-like virus 21	Unclassified	hypothetical protein [Hubei virga-like virus 21]	Blastx	YP_009337659.1	4D	22,8%
Nefer virus	Unclassified	hypothetical protein [Nefer virus]	Blastx	QRW42519.1	1G	20,6%
Formosus virus	Rhabdoviridae	glycoprotein [Formosus virus]	Blastx	WYC12621.1	1D, 1F, 1G, 1H	17,8%

Table 1. Continued

Blast alignment hit	Viral familie	Description	Search strategy	Accessions	Samples	Accession coverage
Beihai narna-like virus 22	Unclassified	RNA-dependent RNA polymerase [Beihai narna-like virus 22]	Blastx	YP_009333139.1	2D, 4G, 4H	14,7%
Partitiviridae sp.	Partitiviridae	putative capsid protein [Partitiviridae sp.]	Blastx	QYW05752.1	1E, 3A	13,6%
Dougludy virga-like virus	Unclassified	putative protein 3 [Dougludy virga-like virus]	Blastx	QI170139.1	4D	10,4%
Aedes anphevirus	Xinnomviridae	putative glycoprotein [Aedes anphevirus]	Blastx	AWW13462.1	1B, 1C, 1E, 1F, 1G, 1H	9,0%
Salarivirus Mos8CMo	Unclassified	nucleocapsid [Salarivirus Mos8CMo]	Blastx	AP161886.1	3E	8,5%
Anopheles flavivirus variant 1	Flaviviridae	Anopheles flavivirus variant 1, complete genome / polyprotein [Anopheles flavivirus variant 1]	Blastn / Blastx	NC_031327.1 / YP_009305197.1	3E, 5E	7,5%
Ochlerotatus caspius flavivirus	Flaviviridae	RNA polymerase [Ochlerotatus caspius flavivirus]	Blastx	CCM73254.1	1D	6,9%
Gurupi chuvirus-like 1	Chuviridae	Gurupi chuvirus-like 1 isolate GcdV1/BR_TO glycoprotein gene, partial cds / glycoprotein [Gurupi chuvirus-like 1]	Blastn / Blastx	MT928173.1 / QPF16715.1	1F	6,4%
Aedes binegev-like virus 2	Unclassified	TPA: hypothetical protein [Aedes binegev-like virus 2]	Blastx	DAZ85684.1	1D, 5F	5,8%
Aedes aegypti anphevirus	Xinnomviridae	glycoprotein [Aedes aegypti anphevirus]	Blastx	QEM39181.1	1D	4,8%

Table 1. Continued

Blast alignment hit	Viral family	Description	Search strategy	Accessions	Samples	Accession coverage
Anopheles flavivirus-like 1	Flaviviridae	polyprotein [Anopheles flavivirus-like 1]	Blastx	AOR51362.1	3F	4.7%
Aedes aegypti To virus 1	Unclassified	nonstructural protein 3 [Aedes aegypti To flavivirus-like]	Blastx	QPF16706.1	1G	3.7%
Karumba virus	Flaviviridae	Karumba virus isolate KRBV Kimi polyprotein gene, complete cds / polyprotein [Karumba virus]	Blastn / Blastx	MF352615.1 / ASD50159.1	3F	3.2%
Burswood narna-like virus	Narnaviridae	RdRp [Burswood narna-like virus]	Blastx	WV103117	2D, 4G	3.2%
Haslams Creek virus	Flaviviridae	polyprotein [Haslams Creek virus]	Blastx	ASD50161.1	3F	2.7%
Hanko totivirus 4	Totiviridae	Hanko totivirus 4 isolate FIN/U-2018/18 Partial RNA dependent RNA polymerase gene, complete cds / RNA dependent RNA polymerase [Hanko totivirus 4]	Blastn / Blastx	OP020055.1 / UUUV42491.1	1D, 1F	2.5%
Chibugado virus	Metaviridae	polyprotein [Chibugado virus]	Blastx	QHA33695.1	1F	2.5%
ssRNA virus-like 6	Unclassified	SsRNA virus-like 6 genomic sequence, sequence	Blastn	KX148585.1	5F	2.1%

Table 1. Continued

Blast alignment hit	Viral familie	Description	Search strategy	Accessions	Samples	Accession coverage
Aedes aegypti To virus 2	Unclassified	Aedes aegypti To virus 2 isolate AaTV2/ BR_TO putative gag protein, putative RNA-dependent RNA polymerase, and putative envelope protein genes, complete cds / putative gag protein [Aedes aegypti To virus 2	Blastn / Blastx	MT913596.1 / QPF16709.1	1F	1,7%
Atrato Virga-like virus 6	Virgaviridae	polyprotein [Atrato Virga-like virus 6]	Blastx	QHA33762.1	4D	1,7%
Anopheles flavivirus variant 2	Flaviviridae	polyprotein [Anopheles flavivirus variant 2]	Blastx	AOR51360.1	3F, 5E	1,6%
Anopheles flavivirus-like 2	Flaviviridae	polyprotein [Anopheles flavivirus-like 2]	Blastx	AOR51363.1	3F	1,2%
Aedes phasmavirus	Phasmaviridae	Aedes phasmavirus strain 17-Lab-adultF RdRp gene, complete cds	Blastn	MT361049.1	1C	0,8%
Orthobunyavirus simbuense	Peribunyaviridae	Simbu virus RdRp gene for RNA-dependent RNA polymerase, segment L, genomic RNA, isolate SA Ar 53	Blastn	NC_018476.1	4F	0,6%
*Ramu Anopheles Virga-like virus	Virgaviridae	MAG: hypothetical protein, partial [Ramu Anopheles Virga-like virus]	Blastx	WPK42831.1	4D	27,3%

Partitiviridae

Partitiviruses are multipartite viruses containing (at least) two dsRNA segments, encoding an RNA-dependent RNA polymerase (RdRp) and a capsid protein, packed in small, isometric, non-enveloped particles [40, 55, 56]. Partitiviruses have an optional third non-structural protein coding segment, referred to as Chaq virus-like RNA segment, which might be part of a satellite-helper virus system or an additional segment of unknown function [40, 55]. The mosquito and *Drosophila* partitiviruses vera virus and galbut virus display very efficient biparental vertical transmission, with maternal transmission reaching 100% and paternal transmission 96 – 100% [55].

In the sampled *Anopheles* mosquitoes from Burkina Faso, partitivirus similarity hits were found in 10 out of the 38 mosquito pools (each containing 12 mosquitoes). Among these viral contigs, we found hits to segment 1 of Partitivirus-like 2 (KX148576.1) and/or Partitivirus-like 3 (KX148577.1). This RdRp encoding segment always co-occurred with Chaq virus-like 2 (KX148555.1) and/or Chaq virus-like 3 (KX148556.1) (Sup3), an optional segment co-occurring with partitiviruses described in *Drosophila melanogaster* [40, 55]. We also found that contig 1G_2721 had a 20.6% coverage of Nefer virus (QRW42519.1), which is also annotated as a Chaq segment [57]. The Chaq segment was proposed to be a satellite virus, a virus which cannot replicate on its own and needs a co-infection to replicate its genome [58]. All our samples containing partitivirus-like segments also contained small RNAs mapping to the Chaq virus-like segment, suggesting it might not be a satellite but rather an extra segment.

BLASTx searches identified the Orestiada partiti-like virus (QRD99858.1), which is a capsid encoding segment, with a 82–100% query coverage and 38.29–66.67% amino-acid identity for contigs 1E_3774, 1F_958, 1F_1999, 1G_258, 1G_720, 1G_3146, 3A_3180, 3D_3206, 3D_3711, 4F_339 and 4F_2572 (Sup3). These capsid segments always co-occurred with RdRP partitivirus-like (and Chaq) sequences, suggesting that these are segments of the same virus. Plotting read sizes showed clear 21 nucleotide peaks for the RdRp, Chaq and capsid segments, corresponding to the typical size of siRNAs, which together with the high read counts is indicative of active replication (Fig2, Sup4).

None of the formed contigs showed the complete genome of the partitivirus RdRp, capsid or the Chaq-segments (Fig2). By repeating contig building on the reads from all pools combined, we were able to build a genome for the partitivirus containing the complete RdRp and Chaq segments and a probably partial capsid sequence (Sup5, Sup6, Sup7), that we named Goden partiti-like virus (GPLV) RdRp, capsid segment and Goden Chaq-like virus segment. Comparing the number of small RNA reads mapping to the GPLV sequences with the reads mapping to our earlier BLAST hits

(Partitivirus-like 2, Partitivirus-like 3, Chaq virus-like 2 and Chaq virus-like 3), we found that more small RNA reads mapped to the newly built genomes for all samples (Sup8). For the newly built genome, the normalized viral read counts (reads per million per segment length) were 2,221 for the RdRp-, 7,263 for the Chaq- and 2,036 for the capsid-segment, yielding an average segment abundance ratio of RdRp : Chaq : capsid of 1 : 3.2 : 0.9. This suggests a relative overabundance of the Chaq-segment and a slight underrepresentation of the capsid with respect to the RdRp. These differences may be due to segment-specific variation in replication dynamics, RNAi machinery recognition, RNA stability, or packaging efficiency.

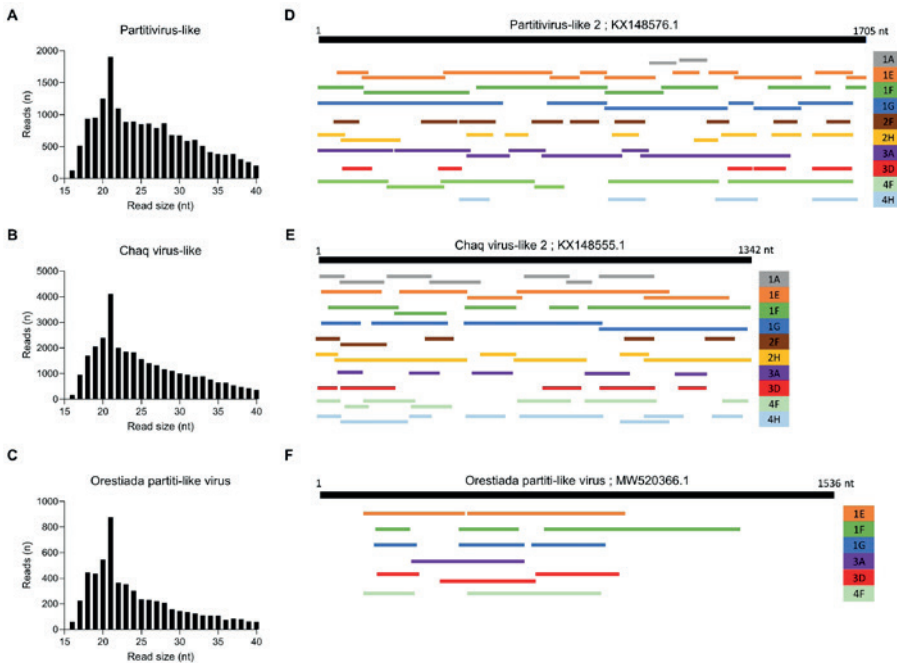


Figure 2. Identification of a novel partitivirus in *Anopheles gambiae*. Size profile of total small RNA reads and schematic presentation of the alignment of contigs to the (A, D) Partitivirus-like RdRp segment, (B, E) Chaq virus-like segment and (C, F) Orestiada partiti-like virus segments.

Several open reading frames (ORFs) could be predicted for each segment using ORFfinder, of which the largest ORF of each segment was used to predict protein structure using AlphaFold3. The RdRp segment (pTM 0.94) was modeled with high confidence with a pIDDT score of over 90 for almost the whole protein, except for short sequences of 8 and 21 amino acids at the N and C-terminus, respectively, showing a typical ‘right-hand’ architecture with subdomains described as fingers, palm and thumb (Fig3a) [59-61]. The capsid segment showed a confident prediction

(pTM 0.83) for most of the structure with a low confident C-terminal tail (Fig3b). 3D cryo-EM of several fungal partitivirus capsids showed capsid dimers with two-fold symmetry whose structures all reflect an assembly of multiple capsid protein subunits, and an ‘arch-like’ surface feature protruding from the capsid shell that is formed by the C-terminal tails [62-66]. It is likely that the GPLV capsid adopts a similar organization. However, predicting the folding of a capsid dimer lowered the pTM score to 0.53 and predicting quaterns resulted in an even lower pTM value of 0.33. A β -sheet structure was predicted with high confidence in the core of the Chaq segment, but we achieved limited reliability for the structure prediction of the majority of the segment (pTM 0.47) (Fig3c).

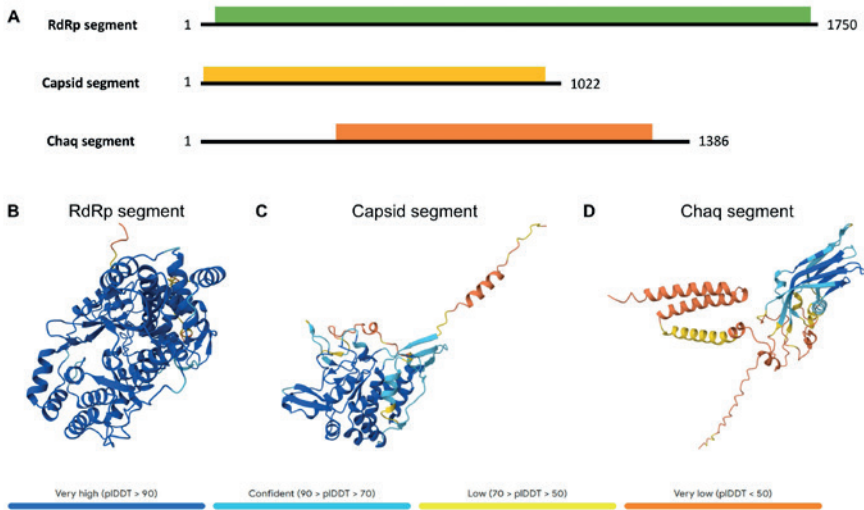


Figure 3. Predictions of Goden partiti-like virus protein structures. (A) Open Reading Frame predictions for the Goden partiti-like virus segments, showing the largest open reading frames with the coloured boxes. (B-D) AlphaFold3 representative 3D predictions of the (A) RdRp-, (B) Capsid- and (C) Chaq proteins of the Goden partiti-like virus protein coding sequence. Color coding corresponds to pIDDT confidence scores.

Phylogenetic comparisons of each partitivirus segment revealed that our GPLV RdRP segment is most closely related to ezimos virus (ONo59772), which is positioned as a small group to a larger, diverse cluster containing Hubei partiti-like virus 14 (KX884163), Hubei partitivirus-like 13 (KX884160), dragana partiti-like virus (MW520412), partitivirus-like 2 (KX148576) and partitivirus-like 3 (KX148577) (Fig4a). The close phylogenetic relationship between GPLV and partitivirus-like 2 and 3 aligns with our previous sequence-similarity searches, further reinforcing that these viruses represent an evolutionary related group. In the capsid-based phylogeny,

Goden partiti-like virus forms a cluster with Nefer virus (MW434499), Beihai barnacle virus 12 ((KX884068)) and Orestiada partiti-like virus (MW520366), although the bootstrap values in that region are relatively modest, the consistent grouping suggests that these viruses share a conserved protein lineage (Fig4b). This is notable as these viruses originate from diverse invertebrate hosts, barnacles and mosquitoes, supporting the idea that the GPLV belongs to a broadly distributed invertebrate-associated clade. Again, in line with our previous sequence-similarity searches, we find a close relationship between the GPLV and the Orestiada capsid sequence. The GPLV Chaq-like sequence clusters with the Zeya Brook chaq-like virus 2 (MW251335), forming a moderately supported lineage (bootstrap 71), within a larger clade that includes Ramu Anopheles Chaq-like virus (OR367400), Zeya Brook chaq-like virus 2 (MW251335) and the Chaq virus-like 2 and 3 (KX148555 and KX148556) (Fig 4c). The phylogenetic trees are dominated by viral sequences identified in arthropods, many of which were originally identified through large metagenomic surveys of insects, ticks and other invertebrates. Some capsid sequences have been found in crustaceans, implying a high viral divergence.

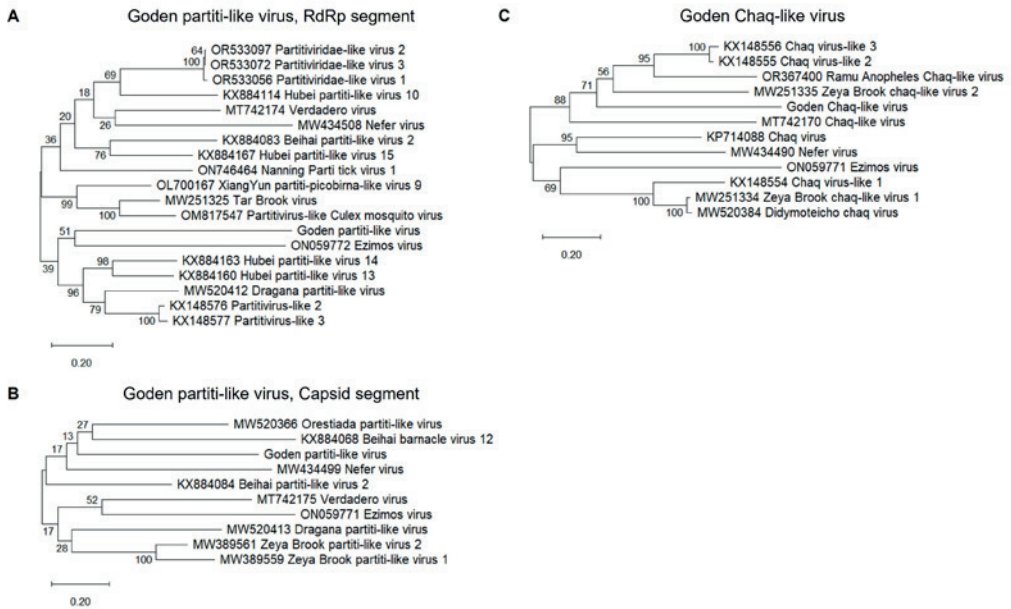


Figure 4. Phylogenetic analysis of the three genomic segments of Goden partiti-like virus. Phylogenetic trees of the (A) RdRp -, (B) Capsid-, and (C) Chaq-segment. The phylogenetic trees are constructed using maximum-likelihood with bootstrapping analysis of 1000 replicates and the number of substitutions per site for the branch length.

Flavivirus-derived Endogenous Viral Element

Flaviviruses are positive-sense, single-stranded RNA viruses, comprising arthropod-borne viruses, transmitted to mammals and birds through arthropod vectors, mainly mosquitoes and ticks [67, 68], as well as insect-specific viruses. Viruses of the *Flaviviridae* family are among the most frequently reported in virus discovery studies in *Anopheles* mosquitoes [49]. In our small RNA datasets, we found contigs with high similarity in BLASTn and BLASTx searches to the *Aedes aegypti* To flavivirus-like virus sequence (MT913594.1) (samples 1D, 1E, 1F, 1G and 1H) with a complete coverage and an identity range of 98.5–100% for each individual contig. The contigs altogether covered 39.6% of the total 816 nucleotide long *Aedes aegypti* To flavivirus-like sequence. The sequence reads mapping to the contigs ranged in size from 26–29 nucleotides, without a peak at 21 nucleotides in each of the eight contigs individually (Sup9.), suggesting that the flavivirus sequences in our data are derived from an *Anopheles* EVE [69–71]. Unexpectedly, a BLASTn search of the *Aedes aegypti* To flavivirus-like sequence (MT913594.1) without taxid restrictions shows a 100% query coverage and a 99.44% nucleotide identity with a genomic locus of *Aedes aegypti* mosquitoes (XM_021848189.1) [72]. The GenBank entry of the *Aedes aegypti* To flavivirus-like viral BLAST hit (MT913594) indicates that the sequence was obtained in a metaviromic analysis of adult *Aedes aegypti* mosquitoes from Tocantins state, Brazil in 2020. However, the earlier described nucleotide sequence XM_021848189.1 corresponding to an *Aedes aegypti* uncharacterized genomic locus found by automated annotation on the *Aedes aegypti* genome in 2017, indicating that this might be an EVE. As the EVE sequence is found in the *Aedes aegypti* genome of 80 male pupae (LVP_AGWG strain), and not in the *Anopheles gambiae* AGAMP3 reference genome [73], the sequences were not removed during our initial genome mapping step. A BLAST search with the *Aedes aegypti* To flavivirus-like as query and the AGAMP3 as database indeed gave no hits. A broader search with all *Culicidae* in the NCBI nucleotide collection (nr/nt database) database only recovered matches to the *Aedes aegypti* XM_021848189.1 locus. Our findings suggest the existence of an EVE in some *An. gambiae* genomes with striking similarity to an EVE in the reference genome of *Ae. aegypti*. The origin of this EVE remains to be identified.

Virgaviridae

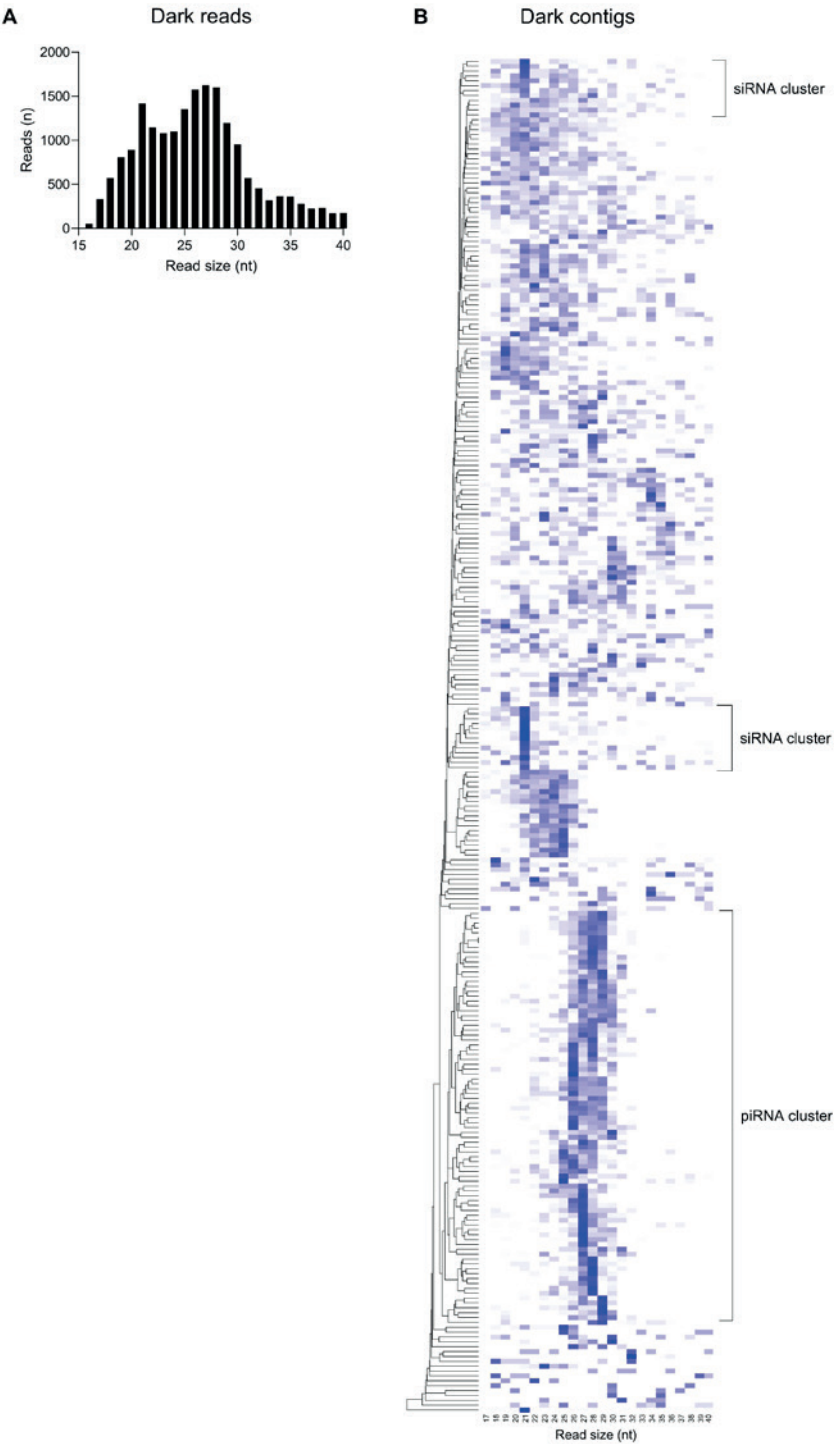
The *Virgaviridae* family consists of rod-shaped, positive-sense single-stranded RNA viruses, traditionally associated with plants and classified into seven genera based on genome organization [74]. Although mosquito virgaviruses have not been formally recognized by ICTV yet, sequences closely related to putative members Hubei virga-like virus 21 (TaxID: 1923336), Atrato virga-like virus 6 (QHA33762.1), and Dougjudy virga-like virus (QIJ70139.1), were identified in sample 4D. While contigs 4D_3912 and

4D_9540 map to Atrato and Dougjudy virga-like viruses, respectively, they did not show significant similarity to Hubei virga-like virus 21 in a virus-restricted BLASTx search. Whilst not identified in an initial analysis in September 2024, a re-analysis of Hubei virga-like virus 21-associated contigs in June 2025 revealed higher similarity (50–100% nucleotide identity) to Ramu Anopheles virga-like virus (TaxID: 3072953), a sequence added to databases in February 2025. Specifically, 8 of 18 Hubei Virga-like virus 21 contigs now show highest similarity to Ramu Anopheles virga-like virus proteins, 5 contigs as the second hit, and 5 still align only with Hubei virga-like virus 21 proteins. Notably, Ramu Anopheles Virga-like virus was identified in a mixture of female *Anopheles spp*, excluding *An. gambiae* and *An. stephensi*, from Bangladesh, while Hubei virga-like virus 21 was originally identified in *An. sinensis* from the Yunnan province, China [75, 76]. As both viruses were only found with a translated nucleotide to protein BLAST, it is likely that our virgavirus sequences are derived from another viral species. Moreover, small RNA size profiles of all virga-like virus mapping contigs do not show a clear 21-nt siRNA, nor a 25–28 nt piRNA peak (Sup10.), and we therefore cannot reliably conclude that these small RNAs derive from a replicating virus.

‘Dark material’

In total, we identified 278 ‘dark contigs’, ranging from 75 to 252 nucleotides and generally displaying a relatively low read coverage ranging from 7 to 189 reads. Clustering the read sizes of these ‘dark contigs’ identified several distinct clades, reflecting different small RNA biogenesis pathways, amongst which clades centered around 21 nt, likely siRNA derived, and 27–28 nt, likely piRNA derived, as well as clades with more dispersed read sizes (Figs). One prominent clade with 27–28 nt reads likely represent piRNAs derived from genomic regions that are absent from the reference genome, perhaps from transposable elements. Of the total of 278 dark contigs, only 21 contigs, divided into two clusters, have a dominant siRNA-like read size of 21 nt and are therefore most likely virus-derived (Fig5B). These might correspond to viruses that are only distantly related to viruses in the database and therefore are not found with our BLAST searches. However, the limited length and coverage of these putative viral contigs precluded us from further characterization.

> **Fig 5. Small RNA profiles of dark material contigs.** (A) Small RNA size profile of all the reads mapping to dark contigs. (B) Heatmap showing the percentage of reads with a specific size over the reads for each dark contig, clustered on profile by average linkage. Clusters with a dominant siRNA-sized peak (21 nucleotides) and a piRNA-sized peak (26–29 nucleotides) are annotated.



Discussion

In this study, we used small RNA sequencing to characterize the virome of *Anopheles gambiae* mosquitoes from Burkina Faso. We found similarity hits to 34 distinct viruses across 10 viral families, amongst which a putative multipartite partitivirus, which we named GPLV, with a high prevalence in the sampled population. In addition, sequences matching a known *Aedes aegypti* EVE were unexpectedly found in the *Anopheles* mosquitoes. Potential *Virgaviridae*-related sequences were found in one sample, however, due to the absence of a clear 21-nt siRNA signature, the origin of this sequence remains unclear. Finally, a small subset of unclassified ‘dark contigs’ showed 21-nt small RNA patterns, indicative for actively replicating viruses, highlighting potential for novel virus discovery using small RNA sequencing.

The most prominent virus in our dataset was the novel GPLV, which appears to be highly prevalent in the sampled *Anopheles* population. Other viruses with *Partitiviridae* family characteristics are found in *Aedes*, *Anopheles* and *Culex* mosquitoes across at least 19 different metagenomic datasets published over the last 20 years [49]. A closely related partitivirus-like segment together with a Chaq-like segment was identified in *Anopheles* spp. from Liberia, Senegal, and Burkina Faso [77], matching the geographic and taxonomic features of our samples. Additionally, Chaq-like segments have been found in five different mosquito studies, of which four report co-infection with a partitivirus RdRp segment [78-82].

Related insect-associated partitiviruses, such as Galbut virus and vera virus in *Drosophila melanogaster* and the Verdadero virus in *Aedes aegypti* suggest a broad insect host range of these viruses [40, 55]. Notably, the capsid sequence is known for only a few insect partitiviruses (Galbut, Vera and Verdadero virus), while capsid sequences are lacking from other mosquito metagenomic studies [49, 55, 78-82]. In our study, a BLASTx hit to the Orestiada partiti-like virus (MW520366.1) allowed us to identify the Goden partitivirus-like capsid. The striking increase in the number of reads mapping to our Goden partitivirus-like capsid compared to the earlier Orestiada partiti-like virus capsid suggests that our capsid sequence is highly divergent from the database reference match. This divergence might be caused by the diversity between host species, *An. Gambiae* and *An. Algeriansis*, as well as geographical separation (Burkina Faso vs. Greece). Unfortunately, attempts to culture the Goden partitivirus *in vitro* (on the *An. gambiae* 4a-3B and mos55 cell lines and the *Aedes albopictus* C6/36 cell line) and *in vivo* (via intrathoracic injection of mosquito lysates into adult *An. stephensi*) were unsuccessful. The relevance of GPLV infections for mosquito physiology and disease transmission therefore remains to be established.

Small RNA sequencing distinguishes replicating viruses and EVEs based on read size, as replicating viruses produce characteristic 21-nt small siRNAs, whereas EVEs often exclusively generate 25 to 29-nt piRNAs [24, 70, 83]. The To flavivirus-like sequence found in our dataset aligned with a 100% query coverage to an *Aedes aegypti* uncharacterized genomic locus (XM_021848189.1), indicating possible EVE origin. The sequence did not occur in the *An. gambiae* reference genome and is even absent from all other *Culicidae* genomes, making it unlikely that the EVE integrated into the genome of an ancestor predating the divergence of these two species. Alternatively, the sequence may have invaded the two mosquito genomes independently. As EVEs can be species- or population-specific, further analyses and experimental validation are required to elucidate the contigs' origin [83].

Similarity based searches, such as BLAST, constrain viral discovery to sequences with homology to existing database entries, which may introduce a detection bias as large, well-characterized families may be overrepresented in sequence databases, increasing their detectability in subsequent searches.

Nevertheless, we found relatively few virus-like contigs with a dominant siRNA-like read size of 21 nt in our 'dark matter' data, suggesting that only few viruses circulate in *Anopheles* that escape detection using similarity searches and that our data accurately represent the virome of our sampled mosquitoes.

A limitation of our study might be sequencing depth, which may affect the reconstruction of complete viral genomes, especially in combination with the short read sizes. While we did not observe a significant correlation between sequence library depth and the number of contigs obtained per sample, several samples with a higher depth (notably 1A-H) generally yielded more contigs than samples with lower depth (2B, 2C, 2E, 4B and 5C). In addition to sequencing depth and read length, lower viral coverage may be due to biological aspects, such as low prevalence of viruses in the sampled mosquito population, low level of viral replication, or a restricted tissue tropism. To address these limitations, long read sequencing, higher sequencing depth and experimental virus isolation could be used to elucidate the *Anopheles* virome in more detail.

Because mosquitoes were collected from a single rural village during a short window in the early wet season, and while this location reflects a typical high-transmission Sahelian ecology, virome composition can differ across ecological contexts, including habitat type and host availability [84]. Temporal fluctuation in mosquito viromes has been reported, with viral prevalence and diversity shifting over the course of the

rainy season due to changes in mosquito age structure and environmental conditions such as temperature, rainfall, and breeding-site turnover [76, 85, 86]. Early wet-season mosquito cohorts are typically younger with fewer cumulative blood meals, whereas later cohorts may accumulate a broader range of horizontally acquired viruses, including arboviruses, over their lifespan. Therefore, our findings represent a snapshot of the local virome, and longitudinal and multi-site sampling would be required to capture the full spatiotemporal heterogeneity of virus circulation in *Anopheles* populations.

Together with other studies using a similar approach [25, 38, 87], our results illustrate that small RNA sequencing is a powerful tool for uncovering both known and novel viruses in wild mosquito populations. The unexpected detection of an *Aedes*-specific EVE in an *Anopheles* population highlights potential genomic integrations that need careful interpretation and more investigation. Additionally, the presence of dark contigs with a 21-nt signature suggest that divergent viruses exist and that complementary approaches, such as long-read sequencing, may be needed to further chart the *Anopheles* virome.

Materials & Methods

Mosquito collection

Anopheles mosquito sampling was carried out at Goden, a rural area located at 40 km in the East of Ouagadougou, the capital of Burkina Faso. Wild adult mosquitoes were collected in houses both in urban and rural area during the period of 8th to 19th August 2022. In the early morning, 5 to 10 houses were visited by two collectors with propack aspirators to collect indoor and outdoor resting mosquitoes. Wild females were knocked down using CO₂ and individually stored at -80 °C until processing.

Sample preparation and library construction

A 96-deepwell plate (CoStar 3958) was filled with 1 mm Zirconica 0.2 g beads (Biospec) using a custom-made aluminium stand with 96 holes of 142 mm² each, to standardize the volume of beads between wells [88]. Tweezers were used to transfer individual frozen mosquitoes to bead-filled wells and subsequently 80 µL PBS was added. Mosquitoes were homogenized for 2 times 10 seconds in a Mini Beadbeater 96 (Biospec), 10 µL of homogenate of 10 to 12 mosquitoes each was transferred to a new tube, to form one pool. Total RNA from each pool was extracted using RNA-Solv reagent (Omega) according to the manufacturer's protocol. Small RNA libraries were constructed with the NEBNext small RNA library kit (New England Biolabs)

and libraries were size-selected on a 6% denaturing PAGE gel followed by ethanol precipitation. The individual small RNA libraries were pooled and sequenced on a NextSeq2000 instrument by GenomeEast (Strasbourg, France) (library 1A–1H) or on an Illumina NextSeq500 instrument (Sequence Facility, Science Faculty, Radboud University, Nijmegen) (library 2A–5F) as 50 nt single-end reads generating an average of ~12 million reads per sample. Sequence data have been deposited in the NCBI sequence read archive, BioProject PRJNA1390778.

Small-RNA sequencing analyses

Small RNA sequence analyses were performed as described by *Aguiar et al.*, 2015 with minor adaptations. Raw sequence reads were submitted to quality filtering and adaptor trimming using FASTQC (v0.12.1) [89] and Clip [90]. Trimmed reads were mapped to the *Anopheles gambiae* reference genome (AgamP3) [73] using Bowtie2 (v2.5.3) [91]. Unaligned reads were used for contig assembly using Velvet (k-mer 15 and default parameters) (v1.2.10) [92]. Contigs > 75 nt were checked for sequence similarity with BLASTn and BLASTx on the NCBI nucleotide collection (nr/nt database, September 2024) restricted for viruses (taxid:10239) [93]. After this, contigs were also analyzed by running BLASTn and BLASTx on the nucleotide collection without taxid restrictions. Assessment if a contig is virus derived, nonviral or 'dark' was done manually. Contigs that did not yield significant BLAST hits in both search strategies were categorized as 'dark'. Contigs with a closest similarity hit to a nonviral sequence in the unrestricted search and no match with a taxid restricted search were categorized as other. Contigs with a closest similarity hit to a virus were categorized as viral, except when the closest hit was a sequence from a synthetic viral construct or phage, which were categorized as other. The coverage of each found accession was shown as the percentage of the length covered by the contigs over the total accession length. Values in the heatmaps show percentages of reads with a specific size of the total aligned reads per contig. Heatmaps were created via Heatmapper, with average linkage for clustering the 'dark' contig small RNAs [94].

As the mosquitoes within and between the pools were derived from the same area and were randomly pooled, we deemed it likely that all partiti/ChaQ sequences were derived from the same virus which we named GPLV. To reconstruct the genome of the Goden partitivirus segments, Partiti-virus mapping small RNA reads of all samples were pooled. RdRp-, Chaq- and capsid-segments were created using Velvet (k-mer 15 and default parameters) (v1.2.10) [92]. The total small RNA reads were then mapped to the partiti-/Chaq-/orestiada capsid sequences (KX148576, KX148577, KX148555, KX148556 and MW520366) and to each of the newly built GPLV segments using Bowtie2 (v2.4.2).

Phylogenetic analysis

The open reading frame (ORF) of each GPLV segment was predicted using ORF-finder [95], and structural motifs in the encoded protein were predicted through AlphaFold3 [96] with piDDT (per residue score predicted confidence) using default settings. Viruses for phylogenetic analyses were selected via a BLASTx similarity search, including one or more viruses with < 90% identity to serve as an outgroup. Viral nucleotide sequences were aligned using MUSCLE in MEGA11 [97]. Phylogenetic trees were reconstructed using maximum likelihood with 1000 bootstraps and ‘Complete Deletion’ of alignment gaps. iTol was used to visualize and optimize phylogenetic tree views [98].

Data storage

Files generated during analyses are stored at the Radboud Repository.

Authors’ contribution

PO and MWG conducted mosquito sample collection in Burkina Faso and together with TB coordinated their shipment to the Netherlands. MS performed sample preparation and carried out the sequencing analyses, including formal data analysis and visualization. MS wrote the original draft of the manuscript. All authors contributed to review and editing and approved the final version of the manuscript.

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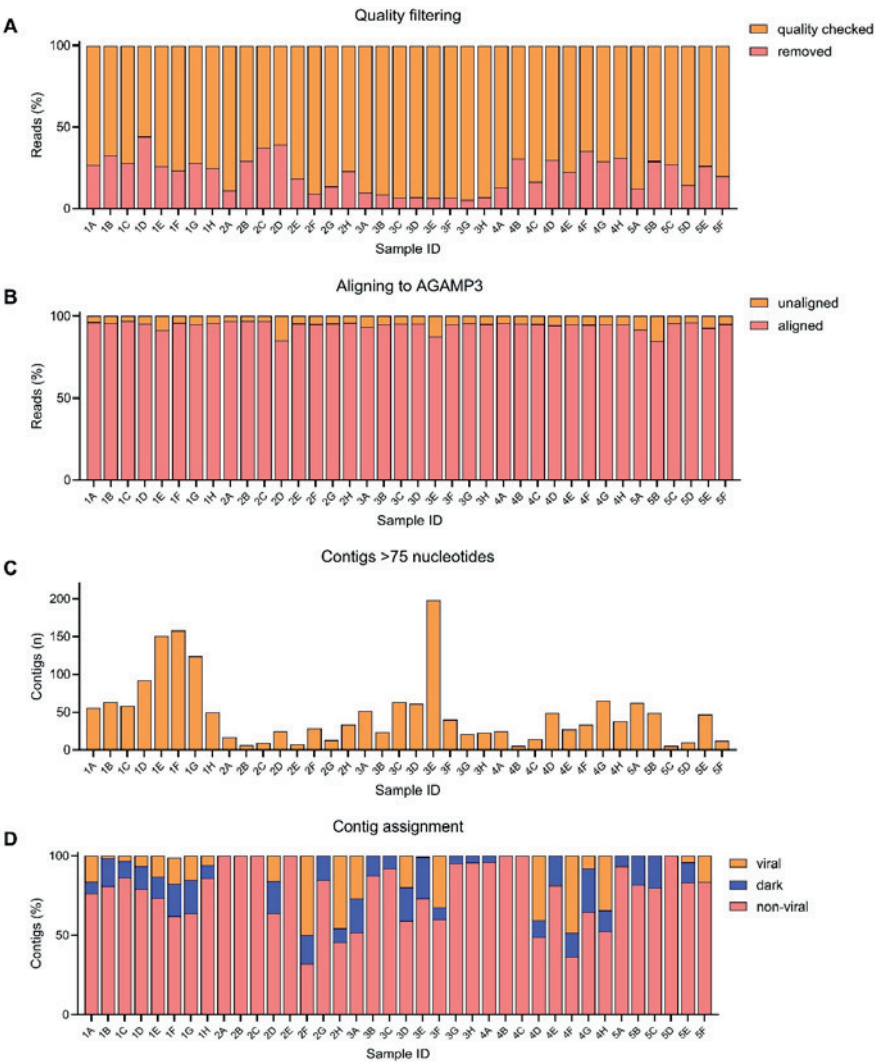
Supplementary materials

S1. Table. General statistics of small RNA analysis. Table showing the number of total small RNA reads per sample, reads after quality checks and trimming, and the reads left after aligning to the *Anopheles gambiae* AGAMP3 reference genome. The number of contigs larger than 75 nucleotides formed per sample and the category (viral, nonviral, dark) are indicated.

Sample number	Sample name	Total reads	Trimmed reads	AGAM unaligned	Contigs >75nt	Viral contigs	Dark contigs	Nonviral contigs
RDVJ733	1A	16826757	12333797	440920	55	9	4	42
RDVJ734	1B	21300719	14361900	584757	63	1	11	51
RDVJ735	1C	16280362	11721429	328670	58	2	6	50
RDVJ736	1D	19019602	10629781	492458	92	6	13	73
RDVJ737	1E	12394273	9181589	790534	151	20	20	111
RDVJ738	1F	20324195	15581583	625304	158	28	32	98
RDVJ739	1G	13092369	9440835	465798	124	19	26	79
RDVJ740	1H	8742406	6583662	274847	50	3	4	43
41250	2A	10179747	9053840	277273	17	0	0	17
41251	2B	10065736	7137280	200805	6	0	0	6
41252	2C	7269176	4552456	129596	9	0	0	9
41253	2D	10159685	6163321	896980	25	3	5	17
41254	2E	4562639	3733261	163383	7	0	0	7
41255	2F	8479210	7724450	372617	28	14	5	9
41256	2G	7817726	6752516	300056	13	0	2	11
41257	2H	9252106	7128768	286545	33	15	3	15
41258	3A	13267342	11982659	787520	52	14	11	27
41259	3B	11709412	10699849	532146	24	0	3	21
41260	3C	19535248	18249880	839455	63	0	5	58
41261	3D	14459715	13477797	618151	61	12	13	36
41262	3E	13033993	12205589	1560327	198	1	51	146
41263	3F	12286917	11487909	565761	40	13	3	24
41264	3G	12077879	11452767	501018	21	0	1	20
41265	3H	9726512	9065177	430984	23	0	1	22
41266	4A	23286407	20240740	822056	25	0	1	24
41267	4B	4537770	3156816	146500	5	0	0	5
41268	4C	11200648	9370791	447315	14	0	0	14

S1. Table. Continued

Sample number	Sample name	Total reads	Trimmed reads	AGAM unaligned	Contigs >75nt	Viral contigs	Dark contigs	Nonviral contigs
41269	4D	14455919	10151033	568480	49	20	5	24
41270	4E	16607547	12894934	643557	27	0	5	22
41271	4F	12520827	8108439	421925	33	16	5	12
41272	4G	18215251	12960228	654639	65	5	18	42
41273	4H	11252130	7780234	388812	38	13	5	20
41274	5A	8988017	7894515	658453	62	0	4	58
41275	5B	9218802	6544862	985142	49	0	9	40
41276	5C	4668244	3396248	146627	5	0	1	4
41277	5D	9073000	7756904	287185	10	0	0	10
41278	5E	7652330	5645854	406200	47	2	6	39
41279	5F	7115510	5712077	272520	12	2	0	10
Total	Total	460656128	362315770	19315316	1812	218	278	1316



S2. Figure. General statistics of small RNA analysis. A) Quality filtering. B) Percentage of reads aligning to the AGAMP3 reference genomes. C) Number of contigs larger than 75 nucleotides. D) Assignment of contigs into viral, nonviral and dark sequences.

S3. Table. Viral hits per contig. Table showing all contigs with a viral hit, alphabetically ordered per virus. For each viral hit, the corresponding sample, contig number, contig length, query coverage, E value, percentage of accession coverage, accession length, and accession number are presented.

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 2	1E	1635	87	Blastn	94,0%	2,00E-28	96,34%	1705	KX148576.1
Partitivirus-like 2	1E	4004	85	Blastn	100,0%	1,00E-28	95,29%	1705	KX148576.1
Partitivirus-like 2	1E	5063	212	Blastn	100,0%	6,00E-57	86,38%	1705	KX148576.1
Partitivirus-like 2	1E	6262	343	Blastn	99,0%	2,00E-143	94,13%	1705	KX148576.1
Partitivirus-like 2	1F	132	333	Blastn	99,0%	7,00E-113	89,43%	1705	KX148576.1
Partitivirus-like 2	1F	870	198	Blastn	98,0%	2,00E-66	90,86%	1705	KX148576.1
Partitivirus-like 2	1F	3178	161	Blastn	89,0%	6,00E-61	97,22%	1705	KX148576.1
Partitivirus-like 2	1G	928	597	Blastn	96,0%	0.0	91,36%	1705	KX148576.1
Partitivirus-like 2	1G	2773	150	Blastn	100,0%	5,00E-46	90,07%	1705	KX148576.1
Partitivirus-like 2	1G	2952	174	Blastn	98,0%	2,00E-51	88,95%	1705	KX148576.1
Partitivirus-like 2	1G	3085	188	Blastn	100,0%	1,00E-73	94,15%	1705	KX148576.1
Partitivirus-like 2	2F	3616	99	Blastn	100,0%	7,00E-33	93,94%	1705	KX148576.1
Partitivirus-like 2	2F	3675	115	Blastn	100,0%	1,00E-36	92,17%	1705	KX148576.1
Partitivirus-like 2	2F	7265	77	Blastn	98,0%	3,00E-20	92,21%	1705	KX148576.1
Partitivirus-like 2	2F	8470	81	Blastn	100,0%	1,00E-29	97,53%	1705	KX148576.1
Partitivirus-like 2	2F	9792	115	Blastn	100,0%	5,00E-40	93,91%	1705	KX148576.1
Partitivirus-like 2	2H	498	76	Blastn	97,0%	2,00E-22	94,59%	1705	KX148576.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 2	2H	4226	99	Blastn	87,0%	3,00E-31	96,55%	1705	KX148576.1
Partitivirus-like 2	2H	5353	86	Blastn	97,0%	1,00E-24	92,86%	1705	KX148576.1
Partitivirus-like 2	2H	5667	93	Blastn	100,0%	6,00E-23	89,36%	1705	KX148576.1
Partitivirus-like 2	3A	3063	143	Blastn	95,0%	4,00E-52	94,89%	1705	KX148576.1
Partitivirus-like 2	3A	3064	238	Blastn	100,0%	5,00E-78	89,50%	1705	KX148576.1
Partitivirus-like 2	3A	3703	236	Blastn	100,0%	1,00E-88	92,37%	1705	KX148576.1
Partitivirus-like 2	3A	6396	253	Blastn	100,0%	5,00E-98	92,89%	1705	KX148576.1
Partitivirus-like 2	3A	16299	116	Blastn	100,0%	3,00E-42	94,83%	1705	KX148576.1
Partitivirus-like 2	3D	15813	75	Blastn	100,0%	9,00E-20	92,00%	1705	KX148576.1
Partitivirus-like 2	4F	952	952	Blastn	98,0%	3,00E-126	94,92%	1705	KX148576.1
Partitivirus-like 2	4F	1567	230	Blastn	96,0%	4,00E-84	92,79%	1705	KX148576.1
Partitivirus-like 2	4H	6604	98	Blastn	97,0%	3,00E-31	93,75%	1705	KX148576.1
Partitivirus-like 2	1A	3993	91	Blastx	98,0%	2,00E-10	96,67%	563	AOR51389.1
Partitivirus-like 2	1A	9196	85	Blastx	88,0%	1,00E-05	80,00%	563	AOR51389.1
Partitivirus-like 2	1E	437	88	Blastx	98,0%	6,00E-10	96,55%	563	AOR51389.1
Partitivirus-like 2	1E	1635	87	Blastx	96,0%	9,00E-09	92,86%	563	AOR51389.1
Partitivirus-like 2	1E	2457	119	Blastx	88,0%	7,00E-14	94,29%	563	AOR51389.1
Partitivirus-like 2	1E	4004	85	Blastx	98,0%	3,00E-11	96,43%	563	AOR51389.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 2	1E	5063	212	Blastx	99,0%	5,00E-44	98.57%	563	AOR51389.1
Partitivirus-like 2	1E	6262	343	Blastx	98,0%	4,00E-73	99.12%	563	AOR51389.1
Partitivirus-like 2	1E	6560	100	Blastx	96,0%	2,00E-13	100.00%	563	AOR51389.1
Partitivirus-like 2	1E	6949	97	Blastx	98,0%	1,00E-13	100.00%	563	AOR51389.1
Partitivirus-like 2	1E	7771	163	Blastx	99,0%	4,00E-28	94.44%	563	AOR51389.1
Partitivirus-like 2	1F	130	411	Blastx	99,0%	1,00E-91	98.53%	563	AOR51389.1
Partitivirus-like 2	1F	177	319	Blastx	98,0%	1,00E-64	96.19%	563	AOR51389.1
Partitivirus-like 2	1F	870	198	Blastx	98,0%	1,00E-23	98.46%	563	AOR51389.1
Partitivirus-like 2	1F	3178	161	Blastx	93,0%	2,00E-22	92.00%	563	AOR51389.1
Partitivirus-like 2	1G	928	597	Blastx	97,0%	3,00E-132	96.41%	563	AOR51389.1
Partitivirus-like 2	1G	1735	389	Blastx	99,0%	2,00E-82	94.57%	563	AOR51389.1
Partitivirus-like 2	1G	1764	84	Blastx	96,0%	3,00E-10	100.00%	563	AOR51389.1
Partitivirus-like 2	1G	2773	150	Blastx	100,0%	2,00E-28	98.00%	563	AOR51389.1
Partitivirus-like 2	1G	3085	188	Blastx	98,0%	8,00E-35	98.39%	563	AOR51389.1
Partitivirus-like 2	2F	2112	99	Blastx	100,0%	2,00E-13	93.94%	563	AOR51389.1
Partitivirus-like 2	2F	3616	99	Blastx	100,0%	2,00E-13	96.97%	563	AOR51389.1
Partitivirus-like 2	2F	3675	115	Blastx	99,0%	6,00E-16	97.37%	563	AOR51389.1
Partitivirus-like 2	2F	7265	77	Blastx	97,0%	1,00E-10	100.00%	563	AOR51389.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 2	2F	8026	94	Blastx	98,0%	4,00E-13	96.77%	563	AOR51389.1
Partitivirus-like 2	2F	8470	81	Blastx	100,0%	1,00E-08	96.30%	563	AOR51389.1
Partitivirus-like 2	2F	9792	115	Blastx	99,0%	9,00E-19	100.00%	563	AOR51389.1
Partitivirus-like 2	2H	498	76	Blastx	94,0%	2,00E-07	100.00%	563	AOR51389.1
Partitivirus-like 2	2H	2716	115	Blastx	99,0%	1,00E-18	100.00%	563	AOR51389.1
Partitivirus-like 2	2H	4226	99	Blastx	87,0%	4,00E-09	89.66%	563	AOR51389.1
Partitivirus-like 2	2H	5330	79	Blastx	98,0%	4,00E-09	100.00%	563	AOR51389.1
Partitivirus-like 2	2H	5667	93	Blastx	96,0%	3,00E-14	100.00%	563	AOR51389.1
Partitivirus-like 2	2H	5807	91	Blastx	95,0%	1,00E-10	93.10%	563	AOR51389.1
Partitivirus-like 2	3A	296	476	Blastx	99,0%	1,00E-105	95.57%	563	AOR51389.1
Partitivirus-like 2	3A	3063	143	Blastx	98,0%	8,00E-25	100.00%	563	AOR51389.1
Partitivirus-like 2	3A	3703	236	Blastx	99,0%	3,00E-43	94.87%	563	AOR51389.1
Partitivirus-like 2	3A	6396	253	Blastx	98,0%	4,00E-50	97.59%	563	AOR51389.1
Partitivirus-like 2	3A	7698	91	Blastx	95,0%	2,00E-12	93.10%	563	AOR51389.1
Partitivirus-like 2	3A	16299	116	Blastx	98,0%	4,00E-18	97.37%	563	AOR51389.1
Partitivirus-like 2	3D	5804	83	Blastx	97,0%	6,00E-10	100.00%	563	AOR51389.1
Partitivirus-like 2	3D	6522	105	Blastx	94,0%	9,00E-16	96.97%	563	AOR51389.1
Partitivirus-like 2	3D	15813	75	Blastx	100,0%	1,00E-07	96.00%	563	AOR51389.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 2	3D	16672	99	Blastx	96,0%	8,00E-10	87,50%	563	AOR51389.1
Partitivirus-like 2	4F	519	98	Blastx	97,0%	4,00E-13	96,88%	563	AOR51389.1
Partitivirus-like 2	4F	539	350	Blastx	99,0%	4,00E-72	93,97%	563	AOR51389.1
Partitivirus-like 2	4F	1567	230	Blastx	96,0%	5,00E-40	93,24%	563	AOR51389.1
Partitivirus-like 2	4F	3592	391	Blastx	96,0%	3,00E-68	96,83%	563	AOR51389.1
Partitivirus-like 2	4H	5672	136	Blastx	99,0%	2,00E-23	100,00%	563	AOR51389.1
Partitivirus-like 2	4H	6604	98	Blastx	97,0%	4,00E-14	100,00%	563	AOR51389.1
Chaq virus-like 2	1A	2028	140	Blastn	100,0%	8,00E-59	97,14%	1342	KX148555.1
Chaq virus-like 2	1A	3685	173	Blastn	100,0%	1,00E-73	96,53%	1342	KX148555.1
Chaq virus-like 2	1A	6277	79	Blastn	98,0%	5,00E-28	97,44%	1342	KX148555.1
Chaq virus-like 2	1E	341	212	Blastn	100,0%	3,00E-85	94,34%	1342	KX148555.1
Chaq virus-like 2	1E	1236	475	Blastn	100,0%	0.0	95,37%	1342	KX148555.1
Chaq virus-like 2	1F	428	165	Blastn	99,0%	1,00E-63	94,51%	1342	KX148555.1
Chaq virus-like 2	1F	1259	92	Blastn	100,0%	1,00E-35	97,83%	1342	KX148555.1
Chaq virus-like 2	1F	1680	520	Blastn	97,0%	0.0	95,46%	1342	KX148555.1
Chaq virus-like 2	1G	764	238	Blastn	100,0%	2,00E-96	94,12%	1342	KX148555.1
Chaq virus-like 2	1G	4439	462	Blastn	100,0%	0.0	96,32%	1342	KX148555.1
Chaq virus-like 2	2F	3614	82	Blastn	91,0%	2,00E-26	97,33%	1342	KX148555.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Chaq virus-like 2	2F	6034	94	Blastn	100,0%	4,00E-35	96,81%	1342	KX148555.1
Chaq virus-like 2	2F	6324	90	Blastn	100,0%	3,00E-26	92,22%	1342	KX148555.1
Chaq virus-like 2	2H	350	91	Blastn	100,0%	3,00E-35	97,80%	1342	KX148555.1
Chaq virus-like 2	2H	3678	85	Blastn	82,0%	1,00E-23	97,14%	1342	KX148555.1
Chaq virus-like 2	3A	101	101	Blastn	100,0%	5,00E-29	91,09%	1342	KX148555.1
Chaq virus-like 2	3A	1339	141	Blastn	100,0%	1,00E-57	96,45%	1342	KX148555.1
Chaq virus-like 2	3A	1873	100	Blastn	96,0%	3,00E-36	96,88%	1342	KX148555.1
Chaq virus-like 2	3D	652	78	Blastn	80,0%	1,00E-19	96,83%	1342	KX148555.1
Chaq virus-like 2	3D	4109	123	Blastn	99,0%	1,00E-50	97,54%	1342	KX148555.1
Chaq virus-like 2	3D	10324	173	Blastn	100,0%	1,00E-73	96,53%	1342	KX148555.1
Chaq virus-like 2	4F	52	224	Blastn	100,0%	3,00E-100	96,88%	1342	KX148555.1
Chaq virus-like 2	4F	120	128	Blastn	100,0%	3,00E-47	94,53%	1342	KX148555.1
Chaq virus-like 2	4F	634	89	Blastn	83,0%	9,00E-26	97,30%	1342	KX148555.1
Chaq virus-like 2	4F	1047	162	Blastn	100,0%	6,00E-66	95,68%	1342	KX148555.1
Chaq virus-like 2	4H	1406	92	Blastn	83,0%	1,00E-25	96,10%	1342	KX148555.1
Chaq virus-like 2	4H	3552	154	Blastn	100,0%	3,00E-68	98,05%	1342	KX148555.1
Chaq virus-like 2	4H	5216	78	Blastn	89,0%	1,00E-23	97,14%	1342	KX148555.1
Chaq virus-like 2	1A	438	144	Blastx	97,0%	4,00E-17	100,00%	298	AOR31385.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Chaq virus-like 2	1A	2028	140	Blastx	98,0%	6,00E-25	100.00%	298	AOR51385.1
Chaq virus-like 2	1E	932	172	Blastx	99,0%	2,00E-33	98.25%	298	AOR51385.1
Chaq virus-like 2	1E	1236	475	Blastx	87,0%	7,00E-72	99.28%	298	AOR51385.1
Chaq virus-like 2	1F	1259	92	Blastx	97,0%	3,00E-12	100.00%	298	AOR51385.1
Chaq virus-like 2	1F	2694	161	Blastx	98,0%	9,00E-30	98.11%	298	AOR51385.1
Chaq virus-like 2	1G	521	430	Blastx	99,0%	5,00E-77	99.30%	298	AOR51385.1
Chaq virus-like 2	2H	350	91	Blastx	95,0%	8,00E-11	100.00%	298	AOR51385.1
Chaq virus-like 2	2H	1549	113	Blastx	98,0%	8,00E-18	97.30%	298	AOR51385.1
Chaq virus-like 2	2H	1778	227	Blastx	99,0%	5,00E-38	98.67%	298	AOR51385.1
Chaq virus-like 2	3A	264	79	Blastx	98,0%	0.031	100.00%	298	AOR51385.1
Chaq virus-like 2	3A	297	128	Blastx	98,0%	2,00E-21	97.62%	298	AOR51385.1
Chaq virus-like 2	3A	1339	141	Blastx	100,0%	2,00E-23	100.00%	298	AOR51385.1
Chaq virus-like 2	3D	4109	123	Blastx	97,0%	1,00E-19	100.00%	298	AOR51385.1
Chaq virus-like 2	4F	52	224	Blastx	89,0%	2,00E-38	98.51%	308	AOR51386.1
Chaq virus-like 2	4F	120	128	Blastx	98,0%	3,00E-20	95.24%	308	AOR51386.1
Chaq virus-like 2	4F	1047	162	Blastx	68,0%	4,00E-17	100.00%	298	AOR51385.1
Chaq virus-like 2	4F	2761	167	Blastx	98,0%	2,00E-23	100.00%	298	AOR51385.1
Chaq virus-like 2	4H	920	261	Blastx	100,0%	3,00E-32	100.00%	298	AOR51385.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Chaq virus-like 2	4H	5216	78	Blastx	88,0%	3,00E-07	100.00%	298	AOR31385.1
Chaq virus-like 3	1A	438	144	Blastn	98,0%	3,00E-53	94.37%	1342	KX148556.1
Chaq virus-like 3	1A	783	81	Blastn	100,0%	5,00E-28	96.30%	1342	KX148556.1
Chaq virus-like 3	1A	1545	161	Blastn	98,0%	6,00E-66	96.23%	1342	KX148556.1
Chaq virus-like 3	1A	2672	141	Blastn	100,0%	2,00E-59	97.16%	1342	KX148556.1
Chaq virus-like 3	1E	932	172	Blastn	99,0%	3,00E-69	95.32%	1342	KX148556.1
Chaq virus-like 3	1E	3139	278	Blastn	96,0%	4,00E-120	96.63%	1342	KX148556.1
Chaq virus-like 3	1E	4409	190	Blastn	100,0%	4,00E-88	98.43%	1342	KX148556.1
Chaq virus-like 3	1F	804	221	Blastn	100,0%	3,00E-100	97.29%	1342	KX148556.1
Chaq virus-like 3	1F	2694	161	Blastn	100,0%	4,00E-67	96.27%	1342	KX148556.1
Chaq virus-like 3	1G	521	430	Blastn	100,0%	0.0	95.12%	1342	KX148556.1
Chaq virus-like 3	1G	541	156	Blastn	90,0%	1,00E-57	96.48%	1342	KX148556.1
Chaq virus-like 3	2F	1827	145	Blastn	100,0%	3,00E-63	97.93%	1342	KX148556.1
Chaq virus-like 3	2F	7148	94	Blastn	100,0%	4,00E-40	100.00%	1342	KX148556.1
Chaq virus-like 3	2H	1421	413	Blastn	100,0%	0,00E+00	95.40%	1342	KX148556.1
Chaq virus-like 3	2H	1549	113	Blastn	100,0%	1,00E-40	94.69%	1342	KX148556.1
Chaq virus-like 3	2H	1778	227	Blastn	100,0%	1,00E-93	94.71%	1342	KX148556.1
Chaq virus-like 3	2H	2598	344	Blastn	97,0%	2,00E-153	96.43%	1342	KX148556.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Chaq virus-like 3	3A	192	80	Blastn	100,0%	4,00E-29	97.50%	1342	KX148556.1
Chaq virus-like 3	3A	297	128	Blastn	100,0%	7,00E-49	95.31%	1342	KX148556.1
Chaq virus-like 3	3D	4318	89	Blastn	100,0%	2,00E-27	93.26%	1342	KX148556.1
Chaq virus-like 3	3D	5150	178	Blastn	100,0%	8,00E-75	96.07%	1342	KX148556.1
Chaq virus-like 3	4F	105	76	Blastn	100,0%	6,00E-27	97.37%	1342	KX148556.1
Chaq virus-like 3	4F	617	94	Blastn	87,0%	2,00E-33	100.00%	1342	KX148556.1
Chaq virus-like 3	4F	2761	167	Blastn	100,0%	1,00E-63	94.01%	1342	KX148556.1
Chaq virus-like 3	4H	920	261	Blastn	100,0%	4,00E-109	94.64%	1342	KX148556.1
Chaq virus-like 3	4H	1129	127	Blastn	100,0%	5,00E-45	93.70%	1342	KX148556.1
Chaq virus-like 3	4H	1457	211	Blastn	100,0%	4,00E-93	96.68%	1342	KX148556.1
Chaq virus-like 3	4H	2061	98	Blastn	100,0%	2,00E-42	100.00%	1342	KX148556.1
Chaq virus-like 3	4H	3838	125	Blastn	93,0%	9,00E-48	97.44%	1342	KX148556.1
Chaq virus-like 3	1A	1545	161	Blastx	98,0%	2,00E-29	98.11%	308	AOR51386.1
Chaq virus-like 3	1A	3685	173	Blastx	91,0%	1,00E-28	98.11%	308	AOR51386.1
Chaq virus-like 3	1E	341	212	Blastx	99,0%	2,00E-42	95.71%	308	AOR51386.1
Chaq virus-like 3	1F	428	165	Blastx	98,0%	2,00E-29	92.59%	308	AOR51386.1
Chaq virus-like 3	1F	804	221	Blastx	65,0%	1,00E-08	95.83%	308	AOR51386.1
Chaq virus-like 3	1F	1680	520	Blastx	37,0%	3,00E-35	98.46%	308	AOR51386.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Chaq virus-like 3	1G	764	238	Blastx	98,0%	4,00E-48	96.15%	308	AOR51386.1
Chaq virus-like 3	1G	4439	462	Blastx	33,0%	9,00E-26	98.08%	308	AOR51386.1
Chaq virus-like 3	2F	6034	94	Blastx	82,0%	4,00E-09	96.15%	308	AOR51386.1
Chaq virus-like 3	2F	6324	90	Blastx	100,0%	6,00E-12	96.67%	308	AOR51386.1
Chaq virus-like 3	2H	1421	413	Blastx	87,0%	1,00E-64	95.83%	308	AOR51386.1
Chaq virus-like 3	3A	1873	100	Blastx	99,0%	1,00E-13	90.91%	308	AOR51386.1
Chaq virus-like 3	3D	5150	178	Blastx	79,0%	5,00E-08	95.74%	308	AOR51386.1
Chaq virus-like 3	3D	10324	173	Blastx	91,0%	1,00E-28	98.11%	308	AOR51386.1
Chaq virus-like 3	4H	1457	180	Blastx	81,0%	1,00E-15	96.49%	308	AOR51386.1
Chaq virus-like 3	4H	3552	154	Blastx	60,0%	2,00E-12	96.77%	308	AOR51386.1
Chaq virus-like 3	4H	3838	125	Blastx	98,0%	7,00E-20	100.00%	308	AOR51386.1
Partitivirus-like 3	1E	1748	87	Blastn	73,0%	7,00E-17	93.75%	1705	KX148577.1
Partitivirus-like 3	1E	2457	119	Blastn	100,0%	1,00E-40	93.28%	1705	KX148577.1
Partitivirus-like 3	1F	164	90	Blastn	73,0%	6,00E-18	93.94%	1705	KX148577.1
Partitivirus-like 3	2F	1287	86	Blastn	82,0%	9,00E-11	85.92%	1705	KX148577.1
Partitivirus-like 3	2F	9308	76	Blastn	100,0%	1,00E-18	90.79%	1705	KX148577.1
Partitivirus-like 3	2H	89	130	Blastn	98,0%	7,00E-44	92.97%	1705	KX148577.1
Partitivirus-like 3	3A	296	476	Blastn	98,0%	6,00E-125	84.29%	1705	KX148577.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 3	3D	6522	105	Blastn	95,0%	2,00E-33	94.00%	1705	KX148577.1
Partitivirus-like 3	3D	7332	125	Blastn	100,0%	3,00E-42	92.80%	1705	KX148577.1
Partitivirus-like 3	4F	3592	391	Blastn	100,0%	2,00E-124	87.76%	1705	KX148577.1
Partitivirus-like 3	4H	3488	143	Blastn	100,0%	8,00E-49	92.31%	1705	KX148577.1
Partitivirus-like 3	1E	640	267	Blastx	98,0%	3,00E-51	95.45%	541	AOR51390.1
Partitivirus-like 3	1F	132	333	Blastx	99,0%	5,00E-69	97.27%	541	AOR51390.1
Partitivirus-like 3	1F	835	188	Blastx	98,0%	9,00E-34	95.16%	541	AOR51390.1
Partitivirus-like 3	1G	2952	174	Blastx	91,0%	2,00E-12	92.45%	541	AOR51390.1
Partitivirus-like 3	2F	1287	86	Blastx	83,0%	2,00E-08	100.00%	541	AOR51390.1
Partitivirus-like 3	2F	9308	76	Blastx	94,0%	2,00E-06	91.67%	541	AOR51390.1
Partitivirus-like 3	2H	89	130	Blastx	57,0%	1,00E-06	92.00%	541	AOR51390.1
Partitivirus-like 3	2H	5353	86	Blastx	97,0%	9,00E-12	100.00%	541	AOR51390.1
Partitivirus-like 3	2H	6249	191	Blastx	98,0%	2,00E-33	95.24%	541	AOR51390.1
Partitivirus-like 3	3A	3064	238	Blastx	98,0%	3,00E-44	97.44%	541	AOR51390.1
Partitivirus-like 3	3D	7332	125	Blastx	60,0%	1,00E-06	92.00%	541	AOR51390.1
Partitivirus-like 3	4F	952	300	Blastx	99,0%	3,00E-63	100.00%	541	AOR51390.1
Partitivirus-like 3	4F	1710	183	Blastx	100,0%	2,00E-31	96.72%	541	AOR51390.1
Partitivirus-like 3	4H	3016	122	Blastx	98,0%	3,00E-20	95.00%	541	AOR51390.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Partitivirus-like 3	4H	3488	143	Blastx	48,0%	3,00E-05	91.30%	541	AOR51390.1
Orestiaa partiti-like virus	1E	3774	419	Blastx	99,0%	4,00E-27	44.29%	430	QRD99858.1
Orestiaa partiti-like virus	1F	958	575	Blastx	87,0%	4,00E-21	38.29%	430	QRD99858.1
Orestiaa partiti-like virus	1F	1999	182	Blastx	82,0%	1,00E-06	47.06%	430	QRD99858.1
Orestiaa partiti-like virus	1G	258	194	Blastx	97,0%	1,00E-07	41.27%	430	QRD99858.1
Orestiaa partiti-like virus	1G	720	196	Blastx	84,0%	4,00E-08	48.21%	430	QRD99858.1
Orestiaa partiti-like virus	1G	3146	128	Blastx	86,0%	2,00E-05	59.46%	430	QRD99858.1
Orestiaa partiti-like virus	3A	3180	287	Blastx	99,0%	1,00E-14	40.62%	430	QRD99858.1
Orestiaa partiti-like virus	3D	3206	227	Blastx	99,0%	1,00E-08	41.33%	430	QRD99858.1
Orestiaa partiti-like virus	3D	3711	250	Blastx	96,0%	1,00E-13	43.21%	430	QRD99858.1
Orestiaa partiti-like virus	4F	339	348	Blastx	96,0%	6,00E-24	45.13%	430	QRD99858.1
Orestiaa partiti-like virus	4F	2572	132	Blastx	100,0%	7,00E-08	56.82%	430	QRD99858.1
Aedes aegypti To flavivirus-like	1D	5409	86	Blastn	100,0%	9,00E-36	100.00%	816	MT913594.1
Aedes aegypti To flavivirus-like	1E	5473	86	Blastn	100,0%	9,00E-36	100.00%	816	MT913594.1
Aedes aegypti To flavivirus-like	1F	178	98	Blastn	100,0%	2,00E-42	100.00%	816	MT913594.1
Aedes aegypti To flavivirus-like	1F	4649	81	Blastn	100,0%	5,00E-33	100.00%	816	MT913594.1
Aedes aegypti To flavivirus-like	1F	9580	95	Blastn	100,0%	5,00E-39	98.95%	816	MT913594.1
Aedes aegypti To flavivirus-like	1G	4551	92	Blastn	100,0%	4,00E-39	100.00%	816	MT913594.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Aedes aegypti To flavivirus-like	1H	3476	132	Blastn	100,0%	9,00E-58	98.48%	816	MT913594.1
Aedes aegypti To flavivirus-like	1D	5409	86	Blastx	97,0%	4,00E-10	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1E	5473	86	Blastx	97,0%	4,00E-10	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1F	178	98	Blastx	97,0%	2,00E-14	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1F	4649	81	Blastx	96,0%	2,00E-09	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1F	9580	95	Blastx	97,0%	7,00E-13	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1G	4551	92	Blastx	97,0%	6,00E-12	100.00%	271	QPF16706.1
Aedes aegypti To flavivirus-like	1H	3476	132	Blastx	97,0%	5,00E-21	95.35%	271	QPF16706.1
Hubei virga-like virus 21	4D	724	130	Blastx	96,0%	3,00E-05	52.38%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	2127	167	Blastx	93,0%	3,00E-05	50.00%	2444	UUG74049.1
Hubei virga-like virus 21	4D	4086	95	Blastx	91,0%	1,00E-06	65.52%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	4291	77	Blastx	97,0%	2,00E-10	92.00%	2444	UUG74049.1
Hubei virga-like virus 21	4D	5349	88	Blastx	92,0%	7,00E-04	55.56%	396	YP_009337661.1
Hubei virga-like virus 21	4D	5566	79	Blastx	94,0%	8,00E-04	76.00%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	5911	82	Blastx	76,0%	0.007	76.19%	2444	UUG74049.1
Hubei virga-like virus 21	4D	5988	123	Blastx	95,0%	4,00E-13	71.79%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	6199	121	Blastx	99,0%	2,00E-06	57.50%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	6415	117	Blastx	100,0%	4,00E-09	70.00%	2444	YP_009337659.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Hubei virga-like virus 21	4D	6985	87	Blastx	96,0%	3,00E-04	71.43%	195	YP_009337660.1
Hubei virga-like virus 21	4D	6990	84	Blastx	92,0%	0.009	65.38%	170	YP_009337662.1
Hubei virga-like virus 21	4D	7492	124	Blastx	96,0%	3,00E-13	72.50%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	7625	99	Blastx	100,0%	7,00E-07	57.58%	2444	UUG74049.1
Hubei virga-like virus 21	4D	8674	85	Blastx	98,0%	3,00E-06	67.86%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	10972	89	Blastx	94,0%	1,00E-10	92.86%	2444	YP_009337659.1
Hubei virga-like virus 21	4D	15177	109	Blastx	99,0%	8,00E-08	66.67%	195	YP_009337660.1
Nefer virus	1G	2721	194	Blastx	95,0%	4,00E-10	43.94%	316	QRW42519.1
Formosus virus	1D	1812	94	Blastx	82,0%	7,00E-09	96.15%	540	DAZ85659.1
Formosus virus	1F	376	84	Blastx	100,0%	1,00E-05	75.00%	540	DAZ85659.1
Formosus virus	1F	978	82	Blastx	98,0%	1,00E-08	88.89%	540	DAZ85659.1
Formosus virus	1F	4572	82	Blastx	98,0%	0.003	66.67%	540	DAZ85659.1
Formosus virus	1F	5831	109	Blastx	99,0%	7,00E-13	80.56%	526	DAZ85662.1
Formosus virus	1F	6515	96	Blastx	87,0%	1,00E-10	96.43%	540	DAZ85659.1
Formosus virus	1F	8443	95	Blastx	97,0%	2,00E-13	100.00%	526	DAZ85662.1
Formosus virus	1G	1144	109	Blastx	85,0%	1,00E-11	93.55%	540	DAZ85659.1
Formosus virus	1G	2774	107	Blastx	92,0%	1,00E-06	75.76%	540	DAZ85659.1
Formosus virus	1H	1224	88	Blastx	96,0%	7,00E-13	96.88%	540	DAZ85659.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Beihai narna-like virus 22	2D	1007	192	Blastx	98,0%	9,00E-21	68.25%	1027	YP_009333139.1
Beihai narna-like virus 22	2D	1966	85	Blastx	98,0%	9,00E-04	64.29%	1027	YP_009333139.1
Beihai narna-like virus 22	4G	1046	77	Blastx	97,0%	7,00E-08	88.00%	1027	YP_009333139.1
Beihai narna-like virus 22	4G	1618	118	Blastx	83,0%	4,00E-06	57.58%	1027	YP_009333139.1
Beihai narna-like virus 22	4G	8688	146	Blastx	67,0%	7,00E-06	63.64%	1027	YP_009333139.1
Beihai narna-like virus 22	4H	82	77	Blastx	66,0%	5,00E-05	88.24%	1027	YP_009333139.1
Partitiviridae sp.	1E	3027	162	Blastx	72,0%	2,00E-05	48.72%	478	QYW05752.1
Partitiviridae sp.	3A	3614	261	Blastx	55,0%	0.003	40.38%	478	QYW05752.1
Dougjudy virga-like virus	4D	9540	156	Blastx	86,0%	3,00E-05	53.33%	382	QI170139.1
Aedes anphevirus	1B	10656	97	Blastx	92,0%	1,00E-10	90.00%	647	AWW13462.1
Aedes anphevirus	1C	1875	94	Blastx	98,0%	4,00E-10	87.10%	647	AWW13476.1
Aedes anphevirus	1E	5665	92	Blastx	97,0%	2,00E-11	90.00%	647	AWW13476.1
Aedes anphevirus	1F	6737	83	Blastx	93,0%	1,00E-04	76.92%	524	AWW13468.1
Aedes anphevirus	1G	1937	96	Blastx	96,0%	4,00E-12	90.32%	647	AWW13476.1
Aedes anphevirus	1H	2926	93	Blastx	96,0%	2,00E-11	90.00%	647	AWW13476.1
Salarivirus Mos8CMo	3E	11762	112	Blastx	99,0%	9,00E-09	67.57%	447	API61886.1
Anopheles flavivirus variant 1	5E	6748	83	Blastn	100,0%	2,00E-32	98.80%	10588	NC_031327.1
Anopheles flavivirus variant 1	3F	3619	93	Blastx	96,0%	5,00E-13	90.00%	3341	YP_009305197.1

S3. Table. Continued

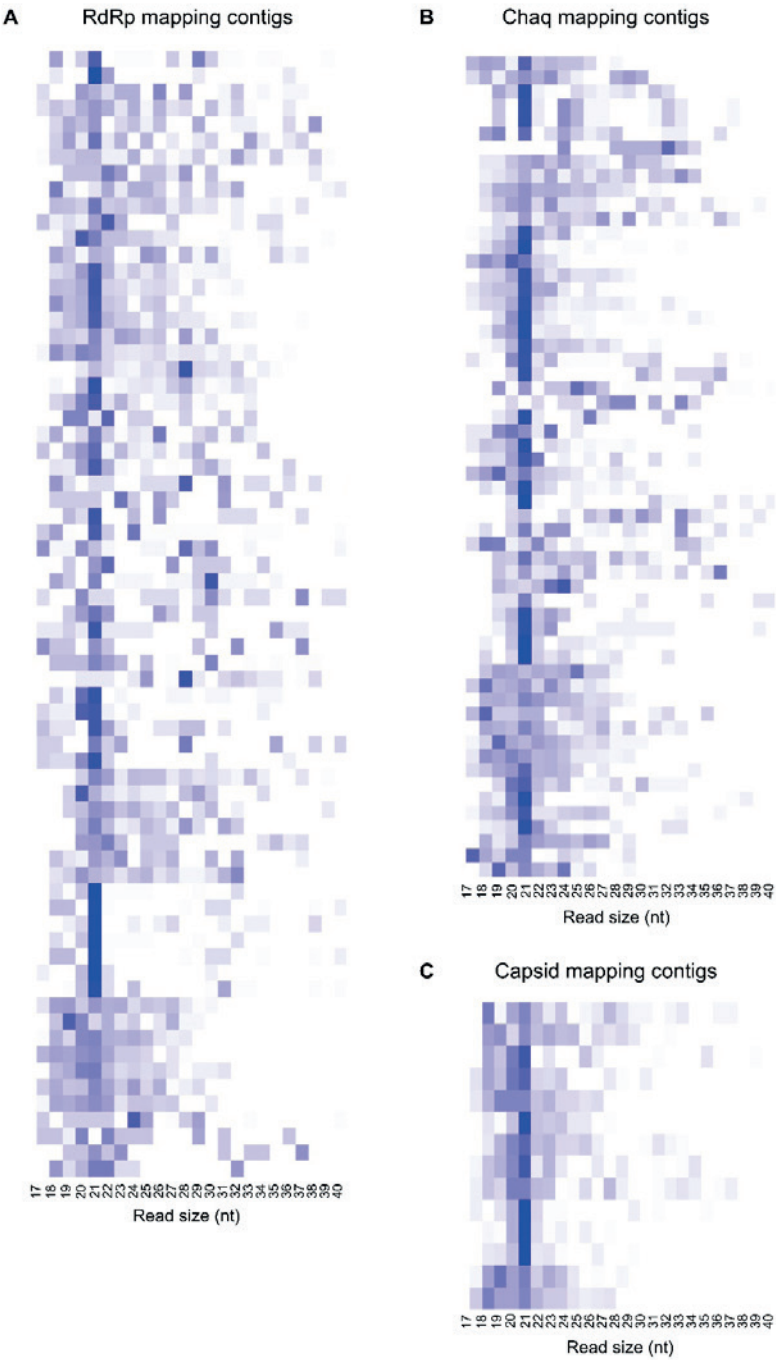
Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Anopheles flavivirus variant 1	3F	6514	81	Blastx	96,0%	6,00E-06	76.92%	3341	YP_009305197.1
Anopheles flavivirus variant 1	3F	7245	107	Blastx	98,0%	1,00E-14	94.29%	3341	YP_009305197.1
Anopheles flavivirus variant 1	3F	9790	174	Blastx	98,0%	5,00E-30	89.47%	3341	YP_009305197.1
Anopheles flavivirus variant 1	3F	10642	96	Blastx	96,0%	8,00E-10	87.10%	385	YP_009305404.1
Anopheles flavivirus variant 1	3F	11273	81	Blastx	85,0%	6,00E-07	91.30%	3341	YP_009305197.1
Anopheles flavivirus variant 1	3F	12637	88	Blastx	98,0%	3,00E-10	86.21%	144	YP_009305409.1
Ochlerotatus caspius flavivirus	1D	3673	75	Blastx	92,0%	2,00E-07	91.30%	321	CCM73254.1
Gurupi chuvirus-like 1	1F	1782	89	Blastn	100,0%	4,00E-34	97.75%	1374	MT928173.1
Gurupi chuvirus-like 1	1F	1782	89	Blastx	97,0%	2,00E-09	96.55%	383	QPF16715.1
Aedes aegypti anphevirus	1D	4521	97	Blastx	98,0%	8,00E-13	90.62%	647	QEM39181.1
Aedes binegev-like virus 2	1D	6226	93	Blastx	96,0%	5,00E-12	96.67%	620	DAZ85684.1
Aedes binegev-like virus 2	5F	7027	75	Blastx	96,0%	0.012	66.67%	2270	DAZ85682.1
Anopheles flavivirus-like 1	3F	3105	92	Blastx	97,0%	2,00E-08	90.00%	2021	AOR51362.1
Aedes aegypti To virus 1	1G	6819	97	Blastx	98,0%	0.008	43.75%	838	QPF16707.1
Karumba virus	3F	19594	122	Blastn	96,0%	3,00E-18	82.20%	10029	MF352615.1
Karumba virus	3F	5029	127	Blastx	96,0%	8,00E-10	60.98%	3342	YP_009388577.1
Karumba virus	3F	21491	77	Blastx	93,0%	9,00E-08	91.67%	3342	ASD50159.1
Burswood narna-like virus	2D	1689	110	Blastx	90,0%	1,00E-05	63.64%	1063	WVLo3117.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
Burswood narna-like virus	4G	3157	91	Blastx	96,0%	3,00E-04	65.52%	1063	WVLO3117.1
Haslams Creek virus	3F	15408	88	Blastx	98,0%	9,00E-11	86.21%	3332	ASD50161.1
Hanko totivirus 4	1D	2186	98	Blastn	100,0%	7,00E-28	91.09%	3794	OP020055.1
Hanko totivirus 4	1F	7473	88	Blastn	100,0%	2,00E-22	90.11%	3794	OP020055.1
Hanko totivirus 4	1D	2186	98	Blastx	85,0%	4,00E-09	86.21%	881	UUV42491.1
Hanko totivirus 4	1F	7473	88	Blastx	88,0%	8,00E-07	85.19%	881	UUV42491.1
Chibugado virus	1F	3882	87	Blastx	96,0%	2,00E-04	67.86%	1081	QHA33695.1
ssRNA virus-like 6	5F	6495	83	Blastn	100,0%	2,00E-32	98.80%	7413	KXI48585.1
ssRNA virus-like 6	5F	7027	75	Blastn	100,0%	4,00E-28	98.67%	7413	KXI48585.1
Aedes aegypti To virus 2	1F	1976	122	Blastn	100,0%	3,00E-47	95.90%	7115	MT913596.1
Aedes aegypti To virus 2	1F	1976	122	Blastx	98,0%	4,00E-19	97.50%	455	QPF16709.1
Atratro Virga-like virus 6	4D	3912	139	Blastx	86,0%	5,00E-04	47.50%	2421	QHA33762.1
Anopheles flavivirus variant 2	3F	16885	91	Blastx	95,0%	3,00E-10	93.10%	3341	AOR51360.1
Anopheles flavivirus variant 2	5E	6748	83	Blastx	97,0%	5,00E-12	100.00%	3341	AOR51360.1
Anopheles flavivirus-like 2	3F	19594	122	Blastx	98,0%	9,00E-20	95.00%	895	AOR51363.1
Aedes phasmavirus	1C	2948	96	Blastn	58,0%	2,00E-12	92.98%	6468	MT361049.1
Orthobunyavirus simbuense	4F	1067	100	Blastn	41,0%	1,00E-10	100.00%	6895	NC_018476.1
Ramu <i>Anopheles Virga-like virus</i>	4D	2127	167	Blastx	95,0%	3,00E-07	50.94%	982	WPK42835.1

S3. Table. Continued

Blast alignment hit	sample	contig	contig length	search strategy	Query Coverage	E value	Percentage Identity	Accession Length	Accession
<i>Ramu Anopheles Virga-like virus</i>	4D	4086	95	Blastx	95,0%	5,00E-07	70.00%	560	WPK42831.1
<i>Ramu Anopheles Virga-like virus</i>	4D	5911	82	Blastx	77,0%	0.026	80.95%	967	WPK42836.1
<i>Ramu Anopheles Virga-like virus</i>	4D	5988	123	Blastx	95,0%	3,00E-13	79.49%	1152	WPK42830.1
<i>Ramu Anopheles Virga-like virus</i>	4D	6199	121	Blastx	99,0%	4,00E-06	60.00%	560	WPK42831.1
<i>Ramu Anopheles Virga-like virus</i>	4D	7492	124	Blastx	97,0%	2,00E-12	77.50%	1152	WPK42830.1
<i>Ramu Anopheles Virga-like virus</i>	4D	7625	99	Blastx	97,0%	4,00E-07	68.75%	982	WPK42835.1
<i>Ramu Anopheles Virga-like virus</i>	4D	10972	89	Blastx	94,0%	3,00E-10	100.00%	1152	WPK42830.1



S4. Figure. *Partitiviridae* heatmaps. Heatmaps showing small RNA read sizes as a the percentage of total reads of each contig for the (A) RdRp, (B) Chaq and (C) capsid segments.

S5. Goden partiti-like virus RdRP segment sequence.

>Goden_partitilike-virus_RdRp_length_1750_cov_1.399261

CACTCAGGGGCTCTCTATATTTATAGTGGGGAGGTTGAATGTGGCCCTTAACACAAGACG
TCATCCTCTATGTCTGGAGGTATGTCGTCTCGTTCCACACCTATCCAGTCGTCGACGCGA
ATGGGATCATCAATGTACTGATAGTACAGGTCCTTAACGTCGGTGACCCTACGAACTGGA
TTTCTCCGGAAGGTTCGGAAGGTGGTCGCATTGGTGCAATTAACCACATCGAAATTTCCG
GGTTGGGGGAAACTGTTAGCGTCCAAGCCATGTAATCTGAGAACTTTAGGCTGTTAGGA
CACTCGGCCATGTGGTCTACAAACCACTGAGGGGAAAGTGAGTACTCGTTCTCAATGCCC
TGGATGATAAGTAACCACCTTTTGGGCGGCTATTCCGTTGAGCGTTGACCACATCTGTCCA
ACCGCGCGAGTAGCAGTAAAACTGGGTCAGGCTCTTGAACCCTCTCAGGTAAACAAAAC
GAAGCTATTAGGAAGTCCTGATCTCTAATGGGATATCCAGAATCGTTATAATAGCCTAAG
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AAGGTCTCACTGGCGACACTAGCTATATCATCAAGGTTAACTATACCCCTTCGTCATAAAC
ACACTATCATCGCCCATGTACAAATCGTACACTGGAAACGACCCTGTAGTTTGGTAGACA
CAGTACCGTGTCACTATGGCGTTTATGATTGAATCGACGATATTAGTGAAGCAGGAACCG
GACGGAACACCGTGTGACTTCATATACCGTGAGCCATCAGGCATACGAAATGGTGTATTT
ATAAAATACTGAACCATTTTCGACCAGCGAGCATTGTGAATATGCGCATTGACTGGCCAC
ACTTTTCCCTCAACATCCACGACGACATCGGTTCTGAAGCACTCCTTCAACAGTGCAAAC
GCATCCCGTACTAACCAAGCAGGGATCTGCTTGTCAAAGCGTCTCCAATCCATCATTACG
GCTTTGACTCCAGGACCAGCACGGTGAAACATCTCATCTACATATCCCATGCCTCCCTTA
CCTATCTCCACACCGTAGGCTAAGGGGTAGTCGTCCCTCCTGTTCTTGAGGAAGTCTAGA
TAAGGGTAAACAAATCTTGCCCTCCTCAATGAACACGCCAGTTGGGTAACCCCATGTAGCG
CGAACCTTGTTGGTACTCGGATCACAAATCTGCGCTCTGGCGTACACGAGACAATCTGGT
AAACGGACGTGATATCCTCTACCGATAAGGATCCAATCCTTTCGAATCTGTCTCATGGCG
TCGTATCAGCGAAGACATCTTCCTTTGTCTTAAAGCCCTGAAGTTCCAGGGTAGACCG
GGAGACTTATCTTTTGGCACCGTTGATACCTTCGAGGCAGCGCCTATCGTTAACGGAATG
ATAGGTACCTGCGGTACAAATTCCTGGCGGACCGATTGTAGAGCAGCAATGTAGATAGGA
TCATCATTGGGTCTTGAAGGCAATTTACAATTATACTCAAACACGTCATCTTGTAATGCA
TCCATTGACACCTTTGACCTATGATACTTTCGAAGAACCCCGACACTACTCGCGATCCA
AATATGAGATTGGAATATCTTACAGCGATCGAGTCCACCTTGGTGGTCTCAAATCTAAT
GGATATCCCTCAGCTGATGGTAACGACTTTATTGGATTACTATCCGTGGAACCTCATTCTA
AAAATGGTTA

S6. Goden partiti-like virus Chaq segment sequence.

>Goden_chaqlike-virus_length_1368_cov_1.730862

CCCAC TTCAACGTTGTGAAGGCCTGAACTTTGCTAAGGTTTCGATTATAGTGGGGGCCAAG
 AAGACAGTACCACGTGTACTCACGCTAAGACGGAGCTTGACGAAGTATGTGGAACGATCA
 ACTCATATTCGCGCGTTTGATATGAACGCTGGGCACGAAGCCGTAGGTGATCAAGTAGAT
 CCTGAACTGGTCTTATGCAACTCACTATGAGTATCATCATCAGGACGATAAGCACGCCAG
 TTGATTGGGTGTGCGGTGGGAGGAGGTTCGGTGGTAGAACACCAGGTGGTAAGTCCTCCTA
 ATGAAGAGAAACTAGTACAGTTAATCTTCATGTCCCTCTCTACACTCTACCGTGAGATCG
 GCGAATGAGCTGAGCGAACCGGAACACAGCGACGCAATGGTGGTAGCGTCACCAACGAGG
 GAATGACGAGCAACTGGATAGACTACGTTAGCAGCGTAGCCGACACCGTCAATGACGGTC
 TTAATGACAGAGTTTGATTCTCTCTGTCCTTCTCCGCTTCTCGACAGCCGCGGCGACT
 TTGTCGATTTCGAGGACCGAGGTTCCGAATCAGTCGCTCAGACTGAGGATAGCTTACGTTT
 GGTGTGGGCGCAGGTAAGCCGTCAGTTCTCCACTCAATCTTTATATGGTACAGGATTGAA
 TCCGTACTCGACGATGATGTGGTTAAGATCTTATACCCGTCGTAACGGGGATCTGGGCGG
 AAGACGCGTCGATGGTGTGATGCTGGATAGTTGTATCCGATTGCCGGGAAGGTGGATGAA
 CCACCACAATCTATCCAAGACCCGAGAGTGTCTTTCTCGGTGTGGTCCGAGTTGCTTATG
 TAATTAATCTCCTCGATGTCCCAAACAACCAAGGAACTAACCTGCCTGTCAGATTCCAA
 ACTGCCACTTTCTGAAAGCGAACTTGACAGTCTGAAAGGCGAACCTTTATCGGAATTCCAG
 TCTGTAGGATTGAAGGTGTTACCCTTACCAACCTGTGCCAGAAAATCAGCGATTAGGTTA
 TTGAAGGTATAGTCGCTGGTTTCCGAATTGCAGATATCGTAACAGGCCACCAAGGTTGA
 GCAGTAACGTCAGGGGGGTTAGGGCCCGGTGAGTACTTACGACCGTAGTCGTTAGCTCGT
 TCAC TTGAGTGTAGTTGGCCTTTGATCTTTTATTACGTTTCTGCTGTACTACCTTCTTC
 TGACTTTTCGATGACATTCTATAACTGCAGTAACTTAAGCCCACGACCTGCGATAGTTC
 GTATAGTAGTCAAGGAAAACTAGCGGGATGAGCTATGTTCAGTTCAC TGTGTTGAACAAAGA
 ATA ACTTCGTCAAGAGAATGTATAACCAGTTTACTTAATAAAATGGTT

S7. Goden partiti-like virus capsid sequence.

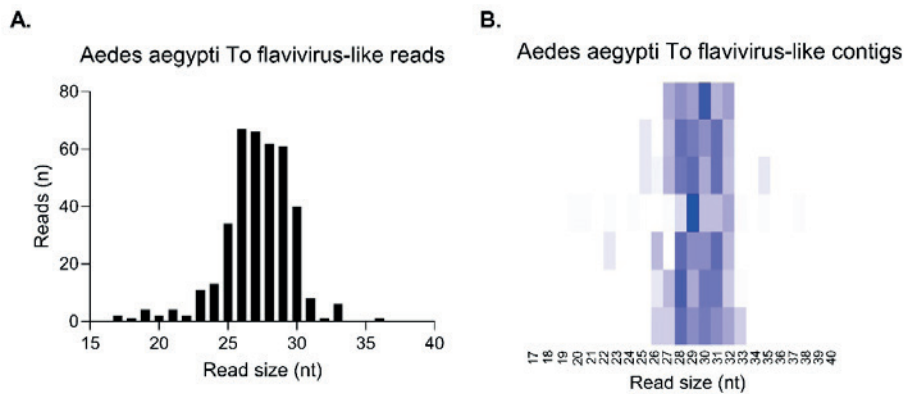
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>Goden_partitilike-virus_capsid_length_1022_cov_1.198883
CCTGGGCACTGGTAAACAGGAACGAGGCCGGCACC CGTTTTC ACTGGGTTGTGACATCC
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GCGACAATGGGCGATGAGCGGGATGCGAAGAGGGCGGTGGAGATGGCACCCACCAGACCT
ACGGCCAACGGAAAACCATGACGGTTTTCCACACCCGTGGTGACGTATGTGATGCTGCGG
TTAGCCACAGTGGCGTAAACCTCAAGATCACGGCACAGTTTGACCTGGCCATCCGTCATC
TTCTTGGAAGGAGTCCAACCAAGTAGATTGTAGTTGGGAACCCCTGGTATGTTCCCTTCC
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ACAGTAACGAGACGCCGCCAGAGCAGTACTATACAGTAGTAGTAGTACTGTGACTCAGAG
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GATGCCCCTTCTATTAATACAGAAGATTTGAGTAGCGGGTCGTTCTTCTCTGCGCTGGGC
GTTGAAGACATAGCCCGAGGTTGGAATGCGACTGCTTGTGTAGCAGTGTCTTCGCGGTCTG
AT

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S8. Table. Small RNA reads mapping to newly built GPLV genome sequences versus the closest BLAST hits

Viral reads per million total reads		partitivirus-like 2	partitivirus-like 3	Goden partitivirus-like		Chaq virus-like 2	Chaq virus-like 3	Goden chaq virus-like		Orestida partitivirus	Goden capsid partitivirus-like
1A		188	118	957		1901	1685	2771		2	481
1E		793	631	3140		3765	3995	6687		1	994
1F		2498	1738	9979		8879	8888	14801		0	2987
1G		1428	1013	5185		8916	8821	14944		0	3519
2F		303	220	1307		762	751	1272		0	424
2H		820	576	2747		5374	4914	8215		0	722
3A		1004	770	4834		10812	11294	20012		1	2014
3D		269	170	1119		796	623	1165		2	1726
4F		2178	1735	8203		15519	14773	26014		28	7644
4H		370	270	1399		2094	2063	3477		0	293



S9. Figure. Flavivirus derived sequences likely derive from an EVE. (A) small RNA size profiles or all the reads mapping to *Aedes aegypti* To flavivirus-like (accession number) and (B) heatmap showing read sizes as a percentage of all reads of each contig mapping to *Aedes aegypti* To flavivirus-like.



S10. Figure. Virgaviridae. Heatmap showing the read sizes as a percentage of all reads of each contig mapping to the Hubei virga-like 21 sequence (accession number).



CHAPTER 4

Comparative analysis of glass and Hemotek membrane feeding systems for malaria transmission research

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Abstract

Background: Glass membrane feeders are used in malaria research for artificial blood feeding. This study investigates the use of Hemotek membrane feeders as a standardized alternative feeding system.

Methods: Hemotek feeders were compared to glass feeders by assessing mosquito feeding rate, imbibed blood meal volume and *Plasmodium falciparum* infection intensity on mosquito guts.

Results: While mosquito feeding rate and blood meal volume was comparable between Hemotek and glass feeders, a loss in transmission was observed using the Hemotek feeder with a conventional collagen membrane; there was no difference in transmission between both feeders when Parafilm was used as the membrane.

Conclusions: Hemotek feeders with a Parafilm membrane can be used as alternative feeding system for malaria transmission research.

Introduction

Malaria remains a major public health problem, with 247 million cases and 619,000 deaths in 2021 [1]. Parasite transmission can occur when *Anopheles* mosquitoes imbibe sexual-stage parasites (gametocytes) that are present in blood of malaria-infected individuals. Membrane feeding assays (MFAs) allow artificial blood feeding of mosquitoes and are fundamental tools in malaria transmission research. Membrane feeding is used for mosquito colony maintenance and for research purposes in the form of direct and standard membrane feeding assays (DMFAs and SMFAs, respectively). DMFAs are commonly used to evaluate the infectiousness of naturally infected gametocyte carriers while SMFAs are the gold standard assay to quantify the capacity of immune factors or compounds to prevent transmission of *in vitro* cultured gametocytes. Malaria MFAs generally use water-jacked membrane feeders from handcrafted glass that are connected to a circulating warm water bath and covered with stretched Parafilm as a feeding membrane. The Hemotek feeding system is a viable alternative that uses electrical heating of metal feeder reservoirs and the blood is covered by a collagen or stretched Parafilm membrane for insects to feed from. Hemotek feeders are commonly used in *Aedes* research for colony maintenance and infection experiments with arboviruses [2], but their current use is limited in malaria research, and sometimes Hemotek systems have been abandoned in favour of glass feeders after observing low *Plasmodium falciparum* infection rates [3]. Compared to custom-made glass feeders, commercial Hemotek feeders have advantages in terms of standardization, temperature control and use as stand-alone systems with a single feeder. However, the Hemotek system has never been validated directly against glass feeders. We therefore compared both feeding systems and assessed the feeding rate of *Anopheles stephensi*, imbibed blood meal volume and performance in *P. falciparum* transmission experiments.

Materials and Methods

An. stephensi mosquitoes (Sind-Kasur strain) were maintained at 30°C and 80% humidity with a reverse day-night cycle (12:12). *P. falciparum* parasites (strain NF135) were cultured in an automated incubator. Venous blood was collected in LiHep BD Vacutainer tubes (#368496; Sanquin, Nijmegen, The Netherlands). Cages containing 50 mosquitoes were exposed for 10 min to feed as described previously [4], using glass midi-feeders or the Hemotek feeding system (PS6120) with 1 mL reservoirs (FU1-1). Parafilm (M laboratory film) or Hemotek collagen (MEM5) were used as feeding membranes. The blood feeding rate was first assessed by visual inspection and

unfed and partially fed mosquitoes were removed. For fully engorged mosquitoes, the blood meal volume was measured in dissected abdomens immediately after feeding using the Hemoglobin Colorimetric Assay Kit (#700540; Cayman Chemical, Ann Arbor, MI, USA) as described previously [4]. Mosquito infectivity was assessed 6-8 d post-infection in a sample of 20 mosquitoes by counting the number of established oocysts in dissected 1% mercurochrome-stained midguts. Linear and negative binomial mixed effects models were used to compare feeding performance and oocysts densities between conditions, accounting for the relatedness of observations from the same experiments.

Results and Discussion

When comparing Hemotek feeders with a collagen membrane against our standard laboratory set-up with glass feeders and Parafilm, we observed no statistically significant difference in mosquito feeding rate (mean of 80% and 78%, respectively; $p=0.94$), or imbibed blood meal volume (median 4.36 μl and 4.09 μl , respectively; $p=0.83$) (Fig. 1A, B). Upon feeding on cultured *P. falciparum* gametocytes, oocyst densities were significantly lower in Hemotek feeders when using the Hemotek collagen membrane ($p<0.0001$) (Fig. 1C). This reduction in transmission efficiency with the collagen membrane may be explained by several factors, including temperature stability, binding of gametocytes by electrostatic forces and the selected feeding membrane. Since the type of feeding membrane has previously been reported as a source of variation in mosquito feeding rate and blood volume [5, 6], we first tested Parafilm as a membrane on Hemotek feeders. For Hemotek feeders, we observed a marked improvement in infection rates for Parafilm compared to collagen membranes ($p<0.001$). There was no statistically significant difference in oocyst density between Hemotek and glass feeders when parafilm was used as membrane ($p=0.44$) (Fig. 1C).

These findings support the use of Hemotek feeders with Parafilm for malaria transmission research. Hemotek feeders are advantageous for small-scale experiments where only a few feeders are needed at the same time. Hemotek feeders are commercially available, do not need specialized glass craftsmanship – thus removing a potential source of variation – and are less fragile. However, in an experimental set-up where a large number of feeders are used concurrently, glass feeders may be preferred since they can be connected in a single chain to the same circulating system, which is more cost effective than multiple Hemotek setups.

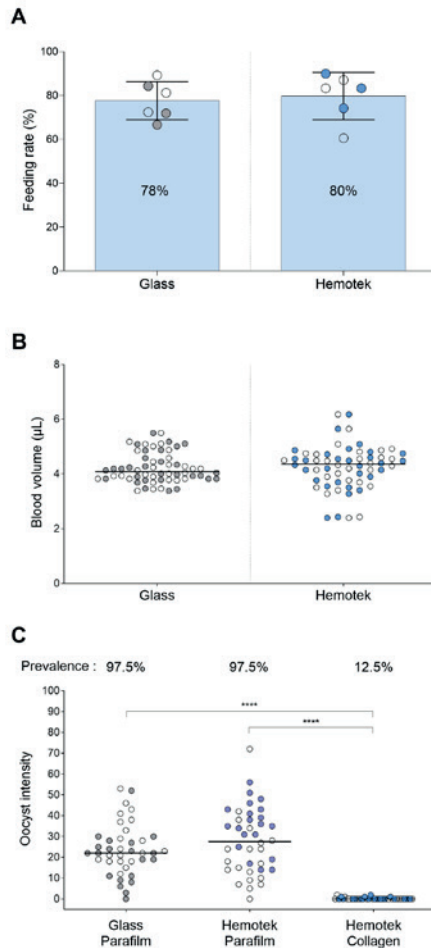


Figure 1. Feeding performance and *P. falciparum* transmission intensity comparing glass against Hemotek membrane feeders. To compare mosquito membrane feeding rate between glass feeders (Parafilm membrane) and Hemotek feeders (collagen membrane), six cups containing 50 mosquitoes were prepared for each setup. Blood feeding was scored by eye and reported as a percentage of total mosquitoes fed. (A) For glass feeders, the mean feeding rate was 77.64% (standard deviation [SD] 8.66, range 66.67-89.29), and for Hemotek feeders it was 79.76% (SD 10.79, range 60.61-90). Subsequently, from each cup 20 fully engorged blood-fed mosquitoes were selected to assess the imbibed blood meal volume. (B) Blood meal volume was not significantly different between glass and Hemotek feeders ($p=0.83$), with a mean volume of 4.23 μL (SD 0.56, range 3.39-5.5) and 4.26 μL (SD 0.79, range 2.4-6.18), respectively. Transmission was assessed in an MFA using cultured *P. falciparum* gametocytes. In duplicate cups per condition, 50 mosquitoes were fed on glass membrane feeders with a Parafilm, or Hemotek feeders with a collagen or Parafilm membrane for 10 min. Infection intensity was measured by the number of developed oocysts 6-8 d post infection on the mosquito midgut, in 20 dissected mosquitoes per feeder. Oocyst densities were significantly lower in Hemotek feeders with collagen membrane when compared with both Hemotek with Parafilm and glass feeder with Parafilm ($p<0.0001$). (C) There was no difference in oocyst density between Hemotek and glass feeder when Parafilm was used as the membrane ($p=0.44$). Duplicate experiments are shown pooled (open and closed circles). **** $P \leq 0.0001$.

Conclusion

We investigated the use of Hemotek membrane feeders as a standardized alternative feeding system for feeding *P. falciparum* gametocytes to *An. stephensi* mosquitoes. We conclude that Hemotek feeders with a Parafilm membrane (but not a collagen membrane) can be used as an alternative feeding system for malaria transmission research.

Authors' contribution

TB, PM, GG, JS, WG and MS conceived and designed the experiments; WG, MS and GG performed laboratory experiments; TB performed data analysis; WG and MS wrote the manuscript together with TB. All authors reviewed, read and approved the final manuscript. TB, WG and MS are guarantors of the paper.

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Competing interests

The authors declare they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Data availability

The datasets used and/or analysed during the present study are available from the corresponding author upon request.

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CHAPTER 5

Systemic infection with insect-specific viruses does not affect *Plasmodium* sporozoite formation in *Anopheles* mosquitoes

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Abstract

Malaria remains a leading cause of morbidity and mortality in large parts of the world. Resistance threatens current preventative and curative strategies, highlighting the need for novel complementary strategies. The opportunities that the mosquito microbiome or virome may offer to reduce pathogen transmission have not been systematically explored for malaria control. In this study, we assessed whether insect-specific viruses affect *Plasmodium* development in mosquitoes. A panel of 17 viruses was tested for *in vitro* and *in vivo* replication in anopheline cells and mosquitoes. From this panel, the RNA viruses Flock House virus, Wallerfield virus, Agua Salud alphavirus, Herbert Herbevirus and Gouléako gouvirus and the dsDNA virus invertebrate iridescent virus 6 efficiently replicated in two *Anopheles gambiae* cell lines and were further assessed in *in vivo* experiments. Intrathoracic injection of these viruses in *Anopheles stephensi* mosquitoes achieved efficient viral RNA replication for Herbert Herbevirus, Agua Salud alphavirus and Gouléako gouvirus and high levels of infectious viral particle production for invertebrate iridescent virus 6. In contrast, Wallerfield virus did not efficiently replicate, and Flock House virus showed high replication, but induced high mosquito mortality. Subsequently, coinfections of the four efficiently replicating, non-lethal insect-specific viruses with the *Plasmodium falciparum* parasite were performed *in vivo*. Sporozoite formation and *Plasmodium* infection rates did not differ between virus-infected mosquitoes and non-infected controls. While none of the tested viruses significantly affected *Plasmodium*, our study identified multiple viruses that efficiently infect *Anopheles stephensi* mosquitoes, providing a useful resource to study *Anopheles* immunity. Future studies may address whether native *Anopheles*-specific viruses affect *Plasmodium* parasite transmission.

Author Summary

Malaria continues to be a major health issue in many endemic countries, and current prevention and treatment methods are not fully effective. This study explores a new way to combat malaria transmission using insect-specific viruses to reduce *Plasmodium* development in mosquitoes. A panel of 17 different insect viruses was tested for replication in mosquito cells and adult female mosquitoes. Four efficiently replicating viruses were subsequently analysed in co-infection experiments, where mosquitoes were first infected with the viruses and subsequently exposed to malaria parasites. However, no significant differences were observed in parasite development, suggesting that persistent infection with these viruses does not affect malaria transmission. Whether other, *Anopheles*-specific viruses affect *Plasmodium* transmission remains to be investigated.

Introduction

Malaria, caused by *Plasmodium* parasites, is a leading cause of morbidity and mortality in many endemic countries. Despite significant progress in lowering disease burden, there were still 263 million malaria cases and 597,000 deaths worldwide in 2023 [1]. Malaria also has a major economic impact with more than 4.3 billion USD governmental spendings worldwide on prevention and treatment per year [2]. Two partially effective malaria vaccines have recently become available for use in endemic regions, which are likely to have a large beneficial public health impact, yet provide incomplete protection from infection and clinical disease [3-5]. Other methods, like widescale availability of effective antimalarial drugs and insecticides on impregnated nets and indoor-spraying have greatly contributed to reduced malaria burden, however, the rise of insecticide resistance in mosquitoes and drug resistance in parasites threatens their effectiveness [6]. Novel, complementary intervention strategies are considered necessary to achieve malaria elimination.

Parasite transmission to mosquitoes starts with the uptake of male and female gametocytes that activate in the mosquito midgut and, as a result of fusion and zygote formation, develop into a motile ookinete. These ookinetes penetrate the mosquito midgut wall to form an oocyst attached to the gut basal lamina that enlarges over time to release sporozoites into the mosquito hemocoel, which then invade the salivary glands [7-11]. At each step of parasite development in the mosquito, there is a considerable loss in parasite numbers and these losses are at least in part attributable to the mosquito immune system [12]. Upon parasite recognition inside the blood meal, hemocytes are recruited and production of reactive oxygen and nitrogen species increases [13, 14]. Subsequently, upon ookinete perturbation of the midgut membrane, the parasite is recognized by pattern recognition receptors that activate the Toll/Rel1 [15], IMD/Rel2 [15-18] and Jak-Stat [19, 20] pathways, leading to the production of antimicrobial peptides (AMPs) like Cecropins, Defensins and Gambicins that can act as antiplasmodial factors [9, 21, 22]. Concurrently, recruited hemocytes initiate processes to lyse or encapsulate the forming oocyst via complement and melanization cascades [23, 24].

Symbiotic microbes may form the basis for innovative intervention strategies to prevent malaria transmission. For example, the formation of oocysts in the midgut is reduced by ~75% if anopheline mosquitoes are colonized with the *Delftia tsuruharens* bacterium [25]. Alternatively, genetically modified *Serratia* or *Pantoea* bacteria are being explored to drive the expression of antimalarial effector proteins in the mosquito midgut to reduce *Plasmodium* infection rates [26, 27]. Antimalarial immune

mechanisms may also be activated by biological agents like bacteria or viruses [28], and prior administration or infection with such an agent may prime the mosquito immune system to more efficiently reduce subsequent *Plasmodium* infection [29].

Anopheles mosquitoes can be infected with a range of insect-specific viruses, which infect mosquitoes but are not transmitted to vertebrates. However, the consequences of systemic virus infection on pathogen transmission have thus far not been studied [29–31]. Evidence from other mosquito species suggest that insect-specific viruses may reduce replication and transmission potential of vector-borne viruses by a mechanism called superinfection exclusion, where prior infection prevents or reduces infection with a second, related virus [32–35]. For example, systemic infection with the insect-specific alphavirus Eilat reduced infection rates at early time points and delayed dissemination of the arthropod-borne chikungunya virus in *Aedes aegypti* [32]. These observations suggest that systemic infection with insect-specific viruses may affect replication of coinfecting pathogens, possible by priming immune responses of the mosquito.

In this study, we analysed whether systemic infection with insect-specific viruses affects *Plasmodium* development in *Anopheles* mosquitoes. We screened a panel of available viruses for *in vitro* replication in two *Anopheles gambiae* cell lines and, for efficiently replicating viruses, *in vivo* in *Anopheles stephensi* mosquitoes. Next, we established an *in vivo* virus-parasite coinfection model in *Anopheles stephensi* to assess viral interference with *Plasmodium* infection. While the selected viruses did not affect sporozoite formation and infectivity rates, the characterized viruses and virus-parasite coinfection model may be useful tools for future studies of *Anopheles* immunity.

Results and Discussion

Screen of insect-specific viruses for replication in *Anopheles* cell lines

In this study, we set out to identify insect-specific viruses that affect replication of the *Plasmodium* parasite in *Anopheles* mosquitoes. While prior knowledge of activation or suppression of mosquito immunity would be useful for selecting candidate viruses, *Anopheles* responses to viral infection had only been studied in the context of infection with the arbovirus O'nyong nyong virus [28]. We therefore took an agnostic approach and screened a diverse panel of viruses, available in the laboratory, for *in vitro* replication in the 4a-3B [36, 37] and mos55 [38] hemocyte-like cell lines, derived from neonate *Anopheles gambiae* larvae. The screen comprised viruses belonging

to 14 different viral genera with diverse genome characteristics and replication strategies. Specifically, we included the positive-sense RNA viruses cell fusing agent virus (CFAV), Kamiti river virus (KRV), cricket paralysis virus (CrPV), Flock House virus (FHV), Wallerfield virus (WALV), Piura virus (PIUV), goutanap virus (GANV), Agua Salud alphavirus (ASALV), Cavally virus (CAVV), Otulum virus (OTUV), Panama sandfly flavivirus (PA-Flavi) and Agua Salud negevirus (ASNV), the segmented, negative-sense viruses Ferak orthoferavirus (FERV), Jonchet orthojonvirus (JONV), Gouléako gouvirus (GOLV) and Herbert herbevirus (HEBV), the bisegmented dsRNA virus *Culex* Y virus (CYV), and the dsDNA virus invertebrate iridescent virus 6 (IIV6) (Table 1). These viruses had been isolated from diverse hosts, including *Aedes* and *Culex* (mosquitoes), *Teleogryllus* (crickets), *Costelytra* (beetles) and *Galleria* (moths) (Table 1). Only GOLV was previously detected in wild-caught *Anopheles* mosquitoes as well as in *Culex* and *Urataenia* spp. mosquitoes [39]. For the other viruses, replication in *Anopheles* had not been previously demonstrated.

Both 4a-3B and mos55 cells were inoculated with each of the insect-specific viruses at 0.01 infectious viral particles per cell (multiplicity of infection), and intracellular and extracellular viral RNA levels were assessed at three days post infection using RT-qPCR. The amount of viral RNA after three days of infection in cells and/or supernatant varied per virus and cell line (Fig. 1A). For nine viruses (OTUV, PA-flavi, FERV, ASNV, GOLV, HEBV, ASALV, WALV and FHV) we detected viral RNA in both the cells and supernatant, indicating active and spreading viral infection. All replicating viruses could use both cell lines as a host, with a general trend of lower replication in the mos55 cell line. For some viruses (KRV, PIUV, GANV, CAVV, CrPV, CYV and JONV), viral RNA was readily detected in the cellular fraction, but limited viral RNA was found in the supernatant, suggesting inefficient viral RNA packaging, virion assembly or release. CFAV was the only virus for which RNA was undetectable both in cells and the supernatant after three days, indicating absent replication. For only one of the viruses, IIV6, visible CPE with cell death and syncytia formation was observed in both 4a-3B and mos55 cells (S1 Fig.). As expected for a DNA virus, no viral RNA was detected in the supernatant, however the clear CPE and syncytia suggest that the virus was actively replicating. Infections of the top-five replicating viruses (FHV, WALV, ASALV, HEBV and GOLV), which had Ct values < 20 for cells and Ct values < 30 for supernatant, and IIV6 were repeated in two additional experiments. A robust increase in viral RNA (FHV, WALV, ASALV, HEBV and GOLV), 10^2 to 10^{10} -fold (Fig. 1B), or titer (IIV6), 10^6 to 10^8 TCID₅₀/mL (Fig. 1C), was seen at three days after infection compared to input.

Table 1. Viruses used in this study. Viruses in bold were analysed in *in vivo* experiments.

Genus	Family	Virus	Host species^a	GenBank accession	Abbr.	Genome	Organization	Envelope	Ref.
Alphamesonivirus	Mesoniviridae	Cavally virus	Aedes barrisoni	GCA_000891195.1	CAVV	(+) RNA	non-segmented	yes	[40]
Alphanodavirus	Nodaviridae	Flock House virus	Costelytra zealandica	GCA_000854385.1	FHV	(+) RNA	segmented	no	[41]
Alphavirus	Togaviridae	Agua Salud virus	Culex declarator	GCA_031192855.1	ASALV	(+) RNA	non-segmented	yes	[42]
Gripavirus	Dicistroviridae	cricket paralysis virus	Antheraea eucalypti	GCA_000853145.1	CrPV	(+) RNA	non-segmented	no	[43]
Entomobirnavirus	Birnaviridae	Culex Y virus	Culex pipiens complex	GCA_031851955.1	CYV	dsRNA	segmented	no	[44]
Flavivirus	Flaviviridae	cell fusing agent virus	Aedes aegypti (cells)	GCA_000862225.1	CFAV	(+) RNA	non-segmented	yes	[45]
Flavivirus	Flaviviridae	Kamiti River virus	Aedes macintoshi	GCA_000851165.1	KRV	(+) RNA	non-segmented	yes	[46]
Flavivirus	Flaviviridae	Panama sandfly flavivirus	-	-	PA-Flavi	(+) RNA	non-segmented	yes	-
Goukovirus	Phenuiviridae	Gouléako goukovirus	Culex nebulosus	GCA_002814655.1	GOLV	(-) RNA	segmented	yes	[39]
Herbevirus	Peribunyaviridae	Herbert herbevirus	Culex nebulosus or Culex decens	GCA_002831165.1	HEBV	(-) RNA	segmented	yes	[47]
Iridovirus	Iridoviridae	invertebrate iridescent virus 6	Chilo suppressalis	GCA_000838105.1	IIV6	dsDNA	non-segmented	yes	[48]
Nelorpivirus	Negevirus	Piura virus	Culex sp.	GCA_002024735.1	PIUV	(+) RNA	non-segmented	no	[49]
Orthoferavirus	Feraviridae	Ferak orthoferavirus	Culex nebulosus or Culex decens	GCA_002814355.1	FERV	(-) RNA	segmented	yes	[50]

Table 1. Continued

Genus	Family	Virus	Host species ^a	GenBank accession	Abbr.	Genome	Organization	Envelope	Ref.
Orthoferavirus	Jonviridae	Jonchet orthojonvirus	Culex decens	GCA_002831065.1	JONV	(-) RNA	segmented	yes	[50]
Sandewavirus	Negevirus	Agua Salud negevirus	-	GCA_031198325.1	ASNV	(+) RNA	non-segmented	yes	-
Sandewavirus	Negevirus	goutanap virus	Culex nebolosus	GCA_000926435.1	GANV	(+) RNA	non-segmented	yes	[51]
Sandewavirus	Negevirus	Wallerfield virus	Culex declarator	GCA_000916075.1	WALV	(+) RNA	non-segmented	yes	[52]

^a Organism from which the virus was first isolated and the associated reference.

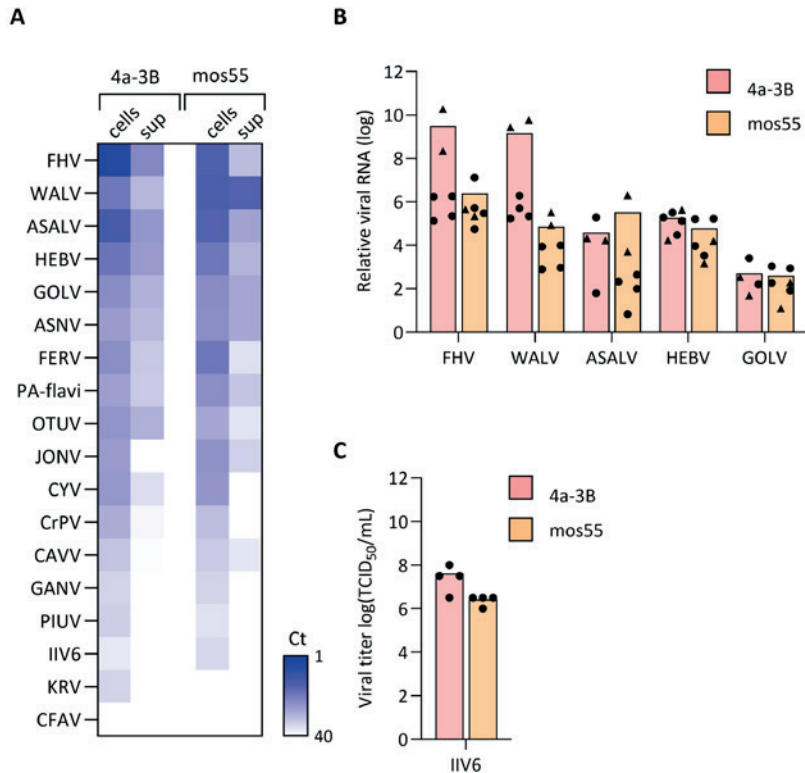


Figure 1. In vitro replication of insect-specific viruses in *Anopheles gambiae* cell lines. (A) Heat map showing Ct values of RT-qPCR for viral RNA in *An. gambiae* 4a-3B (left) and mos55 cells (right) and their culture supernatant at 3 days post infection with the indicated viruses (MOI = 0.01). Data are ordered from top to bottom according to viral RNA levels. IIV6 is a DNA virus and no viral RNA is expected in the supernatant. Data are the mean Ct of duplicate wells in a single experiment. (B) Relative quantification of the top-five viruses from panel A compared to input viral RNA in cells harvested directly after inoculation. Individual data points are shown, with the triangles corresponding to the initial screen and the circles to two additional experiments with duplicate wells, bars indicating the mean +SD. (C) Viral titers of IIV6 infection of two experiments containing duplicate wells, individual data points are shown with bars indicating the mean +SD.

Viral infection does not activate NF-κB pathways in the *An. gambiae* cell lines

Antimalarial defences are regulated by the NF-κB-like Toll/Rel1 and IMD/Rel2 pathways, activation of which leads to the production of AMPs such as Defensin 1 (DEF1, AGAPo11294), Cecropin 1 (CEC1, AGAPoo0693), and Gambicin 1 (GAM1, AGAPoo8645) [9, 21, 22]. We therefore analysed expression of these AMP genes upon viral infection with the top-five replicating viruses and IIV6 at three days post infection. As a positive control, heat-inactivated *E. coli* was used to activate

mos55 and 4a-3B cells. As expected, all three AMP genes were upregulated upon *E. coli* stimulation in both cell lines compared to non-stimulated control (~10 fold). In contrast, no significant upregulation of either AMP was observed in viral infection of *Anopheles* cells (S2 Fig.). Overall, these data suggest that both the 4a-3B and mos55 cell lines are immune competent, as observed previously [36, 37, 53], but do not activate AMP expression upon viral infection.

ASALV, FHV, GOLV, HEBV, and IIV6 efficiently replicate in *Anopheles* mosquitoes *in vivo*

We selected the top-five replicating RNA viruses (FHV, WALV, ASALV, HEBV, GOLV) and the DNA virus IIV6 to investigate viral replication *in vivo* in *An. Stephensi*, a traditional malaria vector in India and invasive in urban settings in Africa [54]. To this end, we inoculated *An. stephensi* mosquitoes by intrathoracic injection and harvested mosquitoes for quantification of viral RNA (FHV, WALV, ASALV, HEBV and GOLV) or infectious titers (IIV6) at three, six-, and nine-days post injection. Consistent with the experiments in the *An. gambiae* cell lines (Fig. 1), FHV, ASALV, HEBV, and GOLV efficiently replicated in *An. stephensi*, reaching 10^3 to 10^5 -fold higher levels of viral RNA compared to the inoculum control, a 100% infection rate, and a replication plateau at three or six days after injection (Fig. 2A, 2C, 2G, 2H). Similarly, IIV6 efficiently replicated *in vivo*, reaching 100% infection rates and peak titers at nine days post infection that were $\sim 10^5$ fold higher than the inoculum (Fig. 2I). Among the viruses tested, WALV was the only one that did not seem to efficiently replicate in *An. stephensi* (Fig. 2B), which was somewhat surprising given efficient *in vitro* replication (Fig. 1). While low levels of viral RNA seemed to accumulate in some mosquitoes, especially at six days post injection, viral RNA levels remained below input levels in over half the mosquitoes. In parallel to assessing viral replication, we monitored mosquito survival (Fig 2D–2F, 2J–2L). FHV infection was highly lethal, inducing 100% mortality between day 3 and 6 (Fig. 2D). In striking contrast, ASALV did not significantly affect survival, even though it accumulated to similar RNA levels as FHV (Fig. 2F). Survival of HEBV, GOLV and IIV6 infected mosquitoes differed significantly compared to PBS injected controls ($P < 0.001$, Log rank Mantel Cox test), and approximately half the mosquitoes survived at 9 days post infection. As expected given its inefficient replication, WALV did not induce mortality (Fig. 2E).

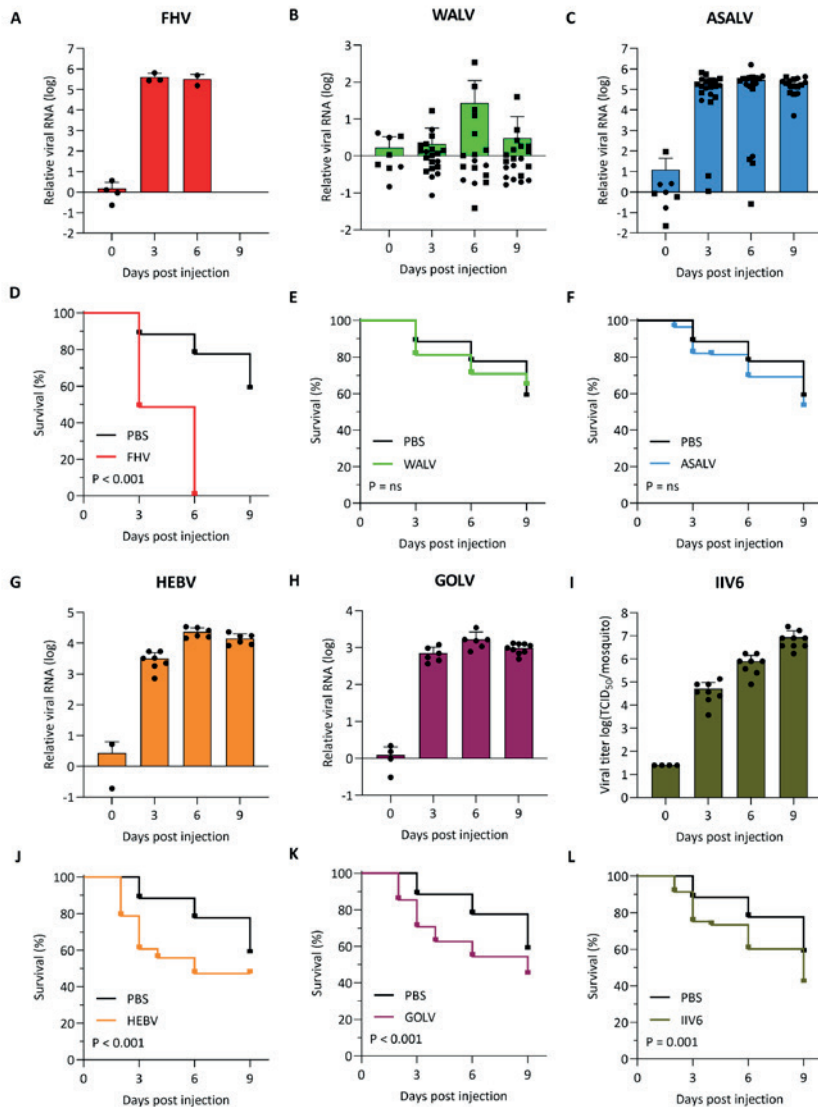


Figure 2. In vivo replication of insect-specific viruses in *Anopheles stephensi* mosquitoes. (A–C, G and H) Relative viral RNA quantification and (I) viral titers for the indicated viruses at 0, 3, 6 or 9 days after intrathoracic inoculation (150 PFU) of adult, female *An. stephensi*. Data points represent individual mosquitoes in a single experiment for FHV, HEBV, GOLV and IIV6 and two experiments for WALV and ASALV (distinguished by circles and squares). All FHV infected mosquitoes were dead at 6 days post injection, viral RNA at that timepoint was quantified in two dead mosquitoes. Bars representing means +SD. (D–F, J–L) Kaplan-Meier survival curves of virus infected mosquitoes and PBS injected control mosquitoes. For clarity, the same curve of the PBS control is shown in all panels. P values from Log-rank Mantel Cox test are indicated, ns = not significant.

Insect-specific viruses do not interfere with *Plasmodium* infections

Given efficient replication and potential for immune activation *in vivo*, we next assessed whether systemic infection with the insect-specific viruses affects *Plasmodium* infection in *An. stephensi* mosquitoes. To establish this *in vivo* virus-parasite coinfection model, we first tested how intrathoracic PBS injection affects blood feeding rates by counting fully fed mosquitoes when a blood meal is offered at 24, 48, 72 and 96 hours after intrathoracic PBS injection. We found that injection reduces feeding rates to approximately 50% compared to noninjected mosquitoes at 24 and 48 h, but blood feeding rates returned to normal at 96 h post injection (86%) (S3 Fig.), which is the time when viral growth is in the log phase or at plateau (Fig. 2). We therefore decided to provide an *P. falciparum* blood meal four days after intrathoracic virus inoculation and to harvest mosquitoes eight days thereafter (Fig. 3A). This time point captures the mid-stage of parasite development in the mosquito midgut, just prior to oocyst rupture and sporozoite migration, when sporozoites in the oocysts can be detected by molecular methods [10, 55].

We analysed sporozoite formation in the virus-parasite coinfection model using ASALV, HEBV, GOLV and IIV6, viruses that efficiently replicated, but, unlike FHV, did not induce overt mortality (Fig. 2). We found that *Plasmodium* infection rates did not differ between virus infected mosquitoes and mock infected mosquitoes (Fig. 3B). Moreover, for all virus infected mosquitoes, the amount of sporozoites detected in individual infected mosquitoes was not significantly different compared to mock infected mosquitoes (Fig. 3C). Conversely, viral RNA levels and infection rates for ASALV, HEBV and GOLV did not significantly differ upon subsequent *Plasmodium* infection compared to blood fed but non-parasite infected controls (Fig. 4A–4C). Interestingly however, viral DNA levels for IIV6 were slightly lower in parasite infected mosquitoes compared to control mosquitoes ($P = 0.0381$, Fig. 4D), suggesting that *Plasmodium* infection creates an environment that may be less supportive of IIV6 replication. To further assess whether there is a correlation between sporozoite numbers and viral RNA or DNA levels, we performed linear regression analyses but found no significant correlations (S4 Fig.). Taken together, our results suggest that systemic infection with these insect-specific viruses does not affect sporozoite formation in *Anopheles* mosquitoes.

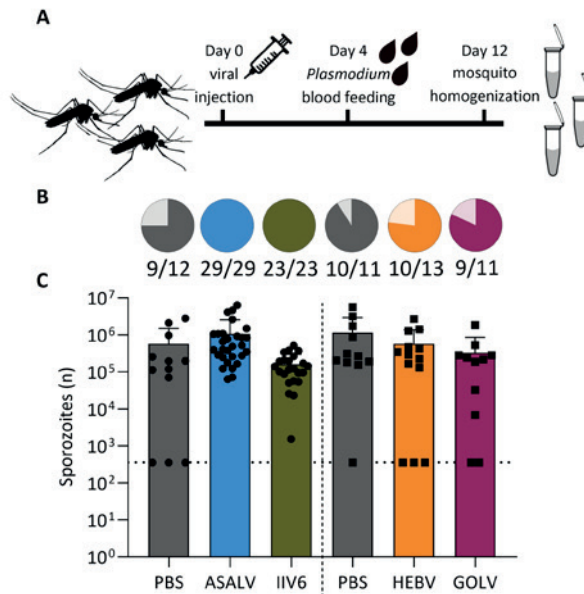


Figure 3. *Plasmodium* infection levels upon viral coinfection in *Anopheles stephensi* mosquitoes.

(A) Outline of the experiment. Mosquitoes were intrathoracically injected with viruses or PBS at day 0. At four days, the injected mosquitoes were offered a *Plasmodium* containing blood meal after which visually fed mosquitoes were selected. At day 12 of the experiment, the mosquitoes were lysed for analysis. (B) infection rates and (C) sporozoite levels in virus infected mosquitoes at 7 days after infectious blood meal (day 12 of the experiment), quantified using RT-qPCR. Original data are shown as points with bars representing means +SD. Data from one experiment including ASALV, IIV6 and PBS control are shown as circles and data from a second experiment including HEBV, GOLV and PBS control are shown as squares. The dotted horizontal line shows the sporozoite detection limit. Negative samples are plotted along the detection limit line, but are not included in the mean. Statistical significance of *Plasmodium* sporozoite levels compared to each internal PBS control was analysed with Kruskal-Wallis and infection rates with Chi-squared tests. No significant differences were found. The mosquito icon was made by Mariana Ruiz Villareal (CCO; bioicons.com/icons/cc-o/Animals/Mariana_RuizVillareal/Mosquito_gender.svg), the syringe icon by Derek Croote (CCO; bioicons.com/icons/cc-o/Lab_apparatus/Derek-Croote/simple-syringe-cartoon.svg), and the tube icon by Andi Wilson (CC-BY 4.0; bioicons.com/icons/cc-by-4.0/Microbiology/Andi-Wilson/Eppendorf_dilutionseries.svg)

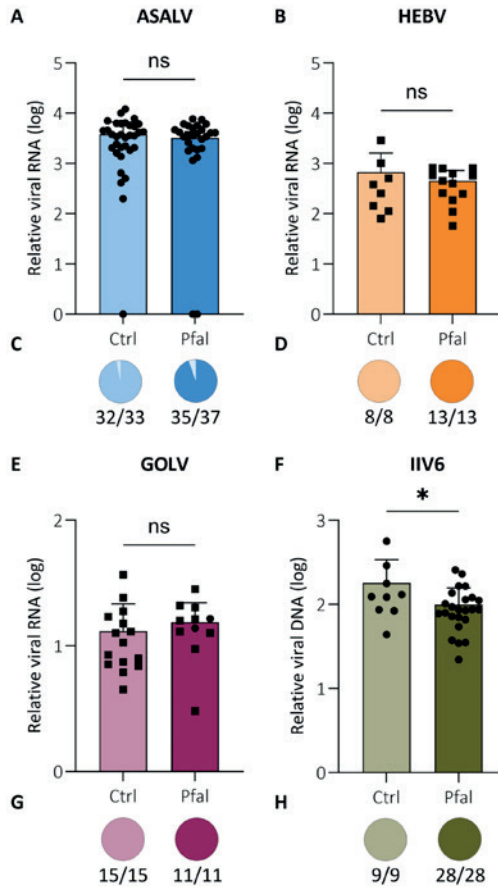


Figure 4. Viral nucleic acids levels upon *Plasmodium* coinfection in *Anopheles stephensi* mosquitoes. Relative viral nucleic acid levels and infection rates of (A,C) ASALV, (B,D) HEBV, (E,G) GOLV and (F,H) IIV6 infected mosquitoes at 7 days after control (ctrl) or *Plasmodium falciparum* (Pfal) infected blood meal. Data for individual mosquitoes are shown as data points, with bars representing means +SD. The circled and square data points represent two batches of blood. Negative samples are plotted along the x-axis, but are not included in the mean. Statistical significance was assessed using unpaired *t*-tests for viral RNA and Chi-squared tests for infection rates. (* $P < 0.05$, ns = not significant)

Conclusion

In this study, we have assessed whether systemic infection with insect-specific viruses interferes with *Plasmodium* development in mosquitoes, potentially affecting malaria transmission. Starting from a screen of 17 viruses, we identified a large number of viruses that replicated in two *Anopheles gambiae* cell lines. We confirmed efficient replication of a subset of these viruses, FHV, WALV, ASALV, HEBV, GOLV and IIV6, in *An. stephensi* mosquitoes *in vivo*. Unfortunately, we found that none of these viruses affected *P. falciparum* sporozoite levels and infection rates.

Our study has several limitations that should be taken into consideration. First, we used *An. stephensi* as our *in vivo* vector model, which may not fully represent the infection and transmission dynamics of *An. gambiae*, the primary malaria vector in many endemic regions. Second, viral infections were established via intrathoracic injection to obtain a consistent, near 100% viral infection rate. This inoculation route bypasses the natural route of infection and may alter virus-vector interactions. In future studies, it will be of interest to test other inoculation routes, especially oral inoculation, which may result in infection of the midgut, the site of *Plasmodium* entry into the mosquito body. Third, with the exception of GOLV, none of the viruses tested have thus far been found to naturally infect *Anopheles* mosquitoes [30]. It will therefore be of considerable interest to analyse native *Anopheles* viruses [30, 31] for *Plasmodium* interference, both from the perspective as a potential biocontrol measure and for understanding natural malaria transmission. Fourth, sporozoite loads were assessed at a time point when sporozoites still reside in mature oocysts, before rupture and salivary gland invasion. These numbers may not accurately reflect numbers of sporozoites expelled from the salivary glands, which would be a more direct readout for transmission potential. As sporozoites still need to migrate to and invade the salivary glands, it remains possible that sporozoite numbers are affected by systemic viral infection at later stages of the infection. Despite these limitations, the tools established in this study will be useful in further dissecting antiviral immunity and the potential interactions between viruses, parasites and mosquitoes.

Methods

Cell culture

The *An. gambiae* mos55 cell line, kindly provided by Jason L. Rasgon (The Pennsylvania State University) [38], the *An. gambiae* 4a-3B cell line (BEI resources, MRA-919) [36, 37], and the *Drosophila melanogaster* S2 cell line [57] were cultured in Schneider's medium

(Gibco) supplemented with 10% heat inactivated fetal calf serum (FCS) (Sigma-Aldrich), 50 U/mL penicillin and 50 µg/mL streptomycin (pen-strep, Sigma-Aldrich). The *Aedes albopictus* C6/36 cell line (ATCC CRL-1660) [57], provided by Sandra Junglen (Charité-Universitätsmedizin Berlin), was cultured in Leibovitz's L-15 medium (Gibco) supplemented with 10% heat inactivated FCS, 2% tryptose phosphate broth solution (Sigma-Aldrich), 1x MEM non-essential amino acids (Gibco), 50 U/mL penicillin and 50 µg/mL streptomycin. All insect cells were incubated at 28 °C without CO₂ supplementation.

Viruses

ASALV, ASNV, CAVV, CYV, FERV, GANV, GOLV, HEBV, JONV, OTUV, PA-flavi, PIUV, and WALV were kindly provided by Sandra Junglen (Charité-Universitätsmedizin Berlin). CFAV (Rio Piedras02 strain) and KRV (SR-75 strain) were provided by the European Virus Archive. CrPV and FHV were kindly provided by Carla Saleh (Pasteur institute) and the IIV6 isolate was kindly provided by Just Vlak (Wageningen University). Viral stocks of CYV, CrPV, FHV and IIV6 were titrated by end-point dilution assays on S2 cells, as described previously [58, 59], and the viral stocks of ASALV, ASNV, CAVV, CFAV, GANV, KRV, OTUV, PA-flavi, PIUV and WALV were titrated on C6/36 cells. Briefly, 1x10⁶ cells/mL were seeded in a 96-well flat-bottom plate, incubated overnight and inoculated with a 10-fold serial dilution of virus samples in quadruplicate. After an incubation of 5–7 days and/or when cytopathic effects (CPE) did not change for two consecutive days, viral titers were calculated as TCID₅₀/mL using the Reed and Muench method [60]. The TCID₅₀/mL values were then converted to plaque forming units (PFU) using the Poisson distribution [61]. Relative genome copies in the viral stocks of FERV, GOLV, HEBV and JONV were quantified by RT-qPCR on a dilution series of viral RNA and used to estimate infectious titers using La-Crosse encephalitis virus stocks with known titers as a reference, as these viruses do not show strong CPE.

Mosquito culture

Anopheles stephensi mosquitoes (Sind-Kasur strain) [62] were reared at 30 °C and 80% humidity with a reverse day–night cycle (12 h:12 h). Adult mosquitoes were maintained at 27 °C and 80% humidity under a day-night cycle (12 h:12 h), with *ad libitum* access to a 10% sucrose solution.

In vitro viral infection

4a-3B and mos55 cells were seeded at a density of 3 x 10⁶ cells/mL in a 24-well plate, incubated overnight, and inoculated at a multiplicity of infection (MOI, the number of infectious viral particles per cell [61]) of 0.01 for each virus in complete cell culture medium. Cells and supernatant were harvested separately at 3 days post infection in 1 mL RNA-solv Reagent (Omega Biotek). Virus inoculum and cells harvested directly

after inoculation were used as input controls. *In vitro* viral infections were performed in three independent experiments with duplicate wells.

Bacterial stimulation

Escherichia coli was cultured overnight on LB-agar plates. Individual colonies were grown in liquid culture of 50 mL LB medium in a shaking incubator to an OD₆₀₀ of 1.0 (corresponding to approximately 8×10^8 cells/mL [63]), heat inactivated at 95 °C for 15 minutes, and stored in aliquots at -80 °C until use. For stimulation experiments, 4a-3B and mos55 cells were seeded at a density of 3×10^6 cells/mL in a 24-well plate, incubated overnight, and 25 µL of a heat inactivated *E. coli* suspension (OD₆₀₀ of 1.0) was added to the medium. Cells were harvested in 1 mL RNA-solv Reagent (Omega Biotek) directly after inoculation (negative control) and after three days.

In vivo viral infection

Three to five-day-old female mosquitoes were used for all experiments. Mosquitoes were infected with the insect-specific viruses via intrathoracic injection of 150 PFUs in a volume of 50 nL, or with 50 nL PBS as a mock infection, using a Nanoject III microinjector (Drummond). Viral replication was assessed at different time points via (RT-)qPCR or end-point dilution assays at 3, 6 and 9 days. Whole mosquitoes were homogenized in 100 µL PBS using a Precellys 24 homogenizer program 5 (Bertin technologies) and stored at -80 °C until titrations (IIV6) or lysed in RNA-solv Reagent (Omega Biotek) and stored in -20 °C until RT-qPCR (FHV, WALV, ASALV, HEBV, GOLV). This was done in one experiment for FHV, HEBV, GOLV and IIV6 and two independent experiments for WALV and ASALV.

Virus-parasite coinfection

Plasmodium falciparum parasites (strain NF135) [64] were cultured in an automated incubator as previously described [65]. Mosquitoes were blood fed four days after viral inoculation. Briefly, cups containing 37–56 mosquitoes were exposed for 15 min to feed on human venous blood collected in LiHep BD Vacutainer tubes (Sanquin, Nijmegen, The Netherlands), with or without *Plasmodium* parasites, using the Hemotek feeding system (PS6120) with 1 mL reservoirs (FU1-1) (Discovery Workshops, UK) and parafilm (M laboratory film) as feeding membrane [66]. Afterwards, mosquitoes were anesthetized on a CO₂ pad, blood feeding rates were assessed by visual inspection, and unfed and partially fed mosquitoes were removed. Viral replication was assessed at different time points via (RT-)qPCR or end-point dilution assays at 12 days post inoculation and *Plasmodium* sporozoite levels were assessed at 7 days post *Plasmodium* infection via (RT-)qPCR. Infection rates were expressed as a percentage or the ratio of the number of infected individuals per total sample population.

Nucleic acid isolation, reverse transcription and qPCR

Cell cultures and homogenized mosquitoes for the *in vivo* viral infections were lysed in RNA-solv Reagent (Omega Biotek) and total RNA was isolated according to manufacturer's instructions. During the coinfection experiments, total nucleic acids (NA) were isolated using the MagNA Pure 96 DNA and Viral NA small volume kit (MagNAPure 96, Roche) according to manufacturer's protocol. For RNA quantifications, samples were treated with DNase I (Invitrogen) before cDNA synthesis using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) according to the manufacturers' protocol. Viral and parasite RNA or DNA levels and host gene expression were measured by qPCR using the GoTaq qPCR Master Mix (Promega) according to manufacturer's instructions on a Light Cycler 480 (Roche). RPL5 was used as the housekeeping gene for *Anopheles* samples. Primer sequences are provided in S5 table. Threshold values (Ct values) were used to calculate the relative RNA levels using the $2^{-\Delta Ct}$ method [67]. For sporozoite counts, a standard curve was derived from a serial dilution of a previously determined in-house sample, and relative counts of the experimental samples were determined using the fitted curve. Negative samples in bar graphs were plotted as values at the detection limit, but these values were not used to calculate the mean.

Statistical analyses

Survival curves were generated using the Kaplan-Meier nonparametric estimation from censored data, and statistical significance was determined using Log rank Mantel Cox tests [68]. Differences in AMP expression were examined with one-way ANOVA for multiple comparisons and sporozoite counts with Kruskal-Wallis tests, both of each sample group against its internal control. Viral RNA or DNA infection levels were analysed using unpaired *t*-tests on log transformed data. Infection rates were compared using Chi-squared tests, and correlations were analysed using the Pearson correlation method. All statistical tests were performed using GraphPad Prism 10.

Authors' contribution

MS, PM, TB and RR contributed to the conceptualization of the study. SJ facilitated the use of several viruses. MS performed the investigation, including the initial *in vitro* screening and all subsequent *in vitro* experiments. EP performed the experiments including antimicrobial peptides under supervision of MS. The *in vivo* experiments were conducted by MS with support from GJG, PM and GO. MS carried out the formal data analysis and wrote the original draft of the manuscript. All authors contributed to review and editing and approved the final version of the manuscript.

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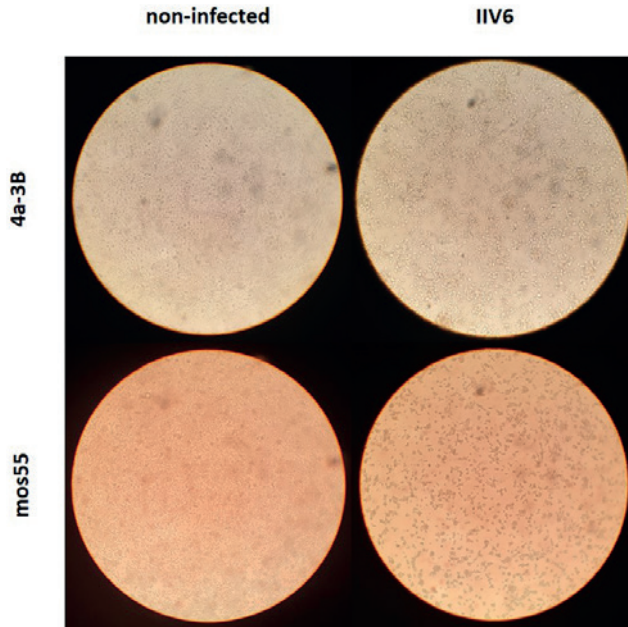
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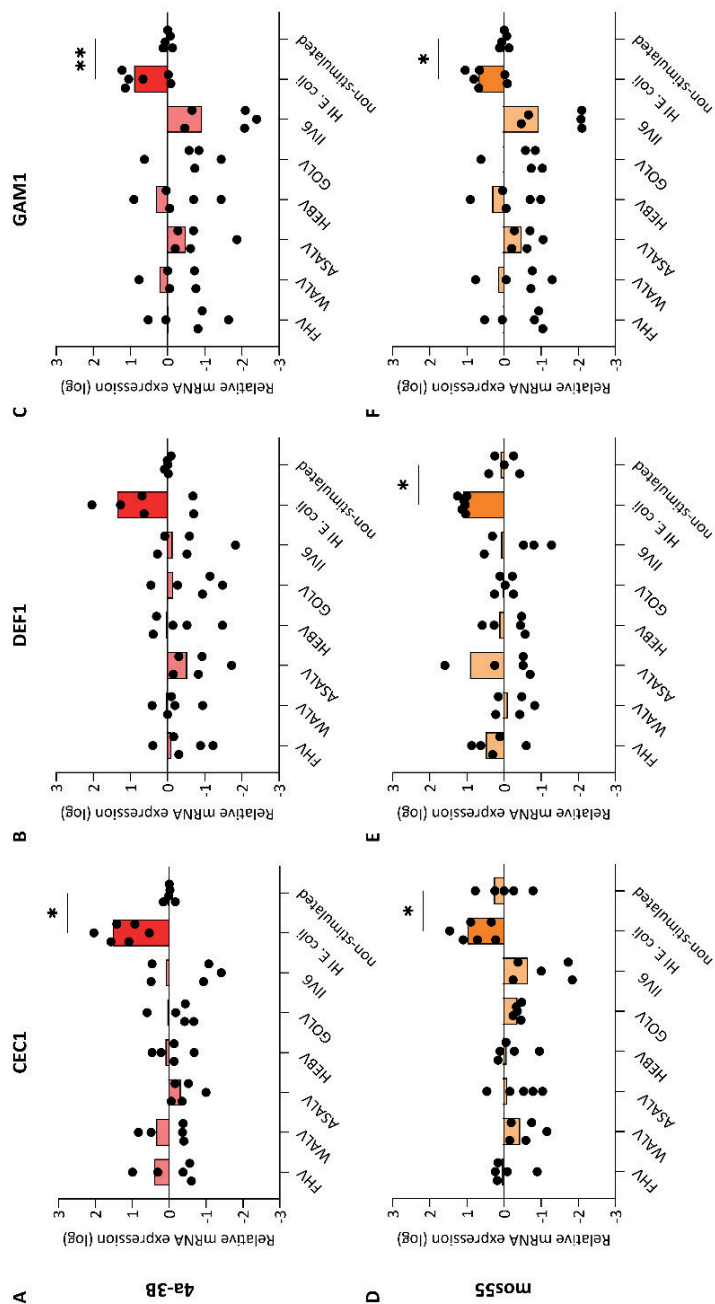
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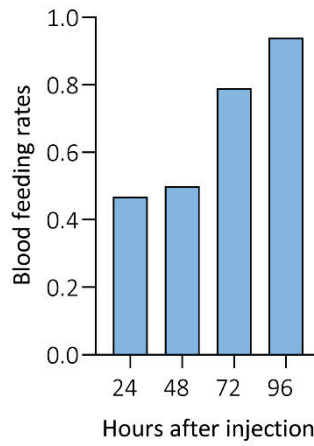
Supplementary Materials



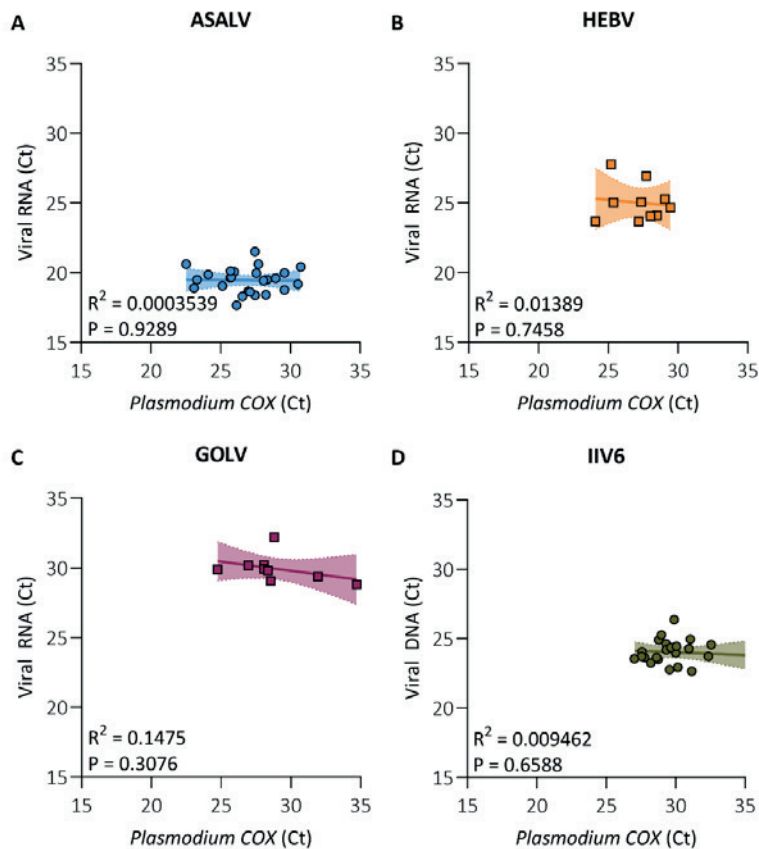
S1 Fig. IIV6 infection in *Anopheles gambiae* cells causes cytopathic effect. Microscopy image of uninfected (left) and IIV6 infected (right) 4a-3B and mos55 cells.



S2 Fig. AMP induction of insect-specific viruses in *Anopheles gambiae* cell lines. Relative mRNA expression of the AMP genes (A, D) *CEC1*, (B, E) *DEF1* and (C, F) *GAM1* in (A - C) 4a-3B and (D - F) mos55 cell lines at 3 days after viral infection (FHV, WALV, ASALV, HEBV, GOLV, IIV6) at an MOI of 0.01 or after stimulation with heat inactivated (HI) *E. coli*. Expression was measured by RT-qPCR and expressed relative to expression in non-stimulated cells. Bars indicating the mean of three experiments with duplicate wells for all *E. coli* stimulations and two duplicate wells and one individual well for the viruses and non-stimulated control, individual data points shown as dots. Statistical significance of each stimulated or infected group compared to non-stimulated control was analysed with one-way ANOVA. (***) $P < 0.005$, (*) $P < 0.05$



S3 Fig. Blood feeding rates of *Anopheles stephensi* mosquitoes after intrathoracic injection. Bars represent the ratio of the percentage of blood-fed PBS injected mosquitoes over the percentage of blood-fed non-injected control mosquitoes with 20 to 27 mosquitoes within each group in one experiment.



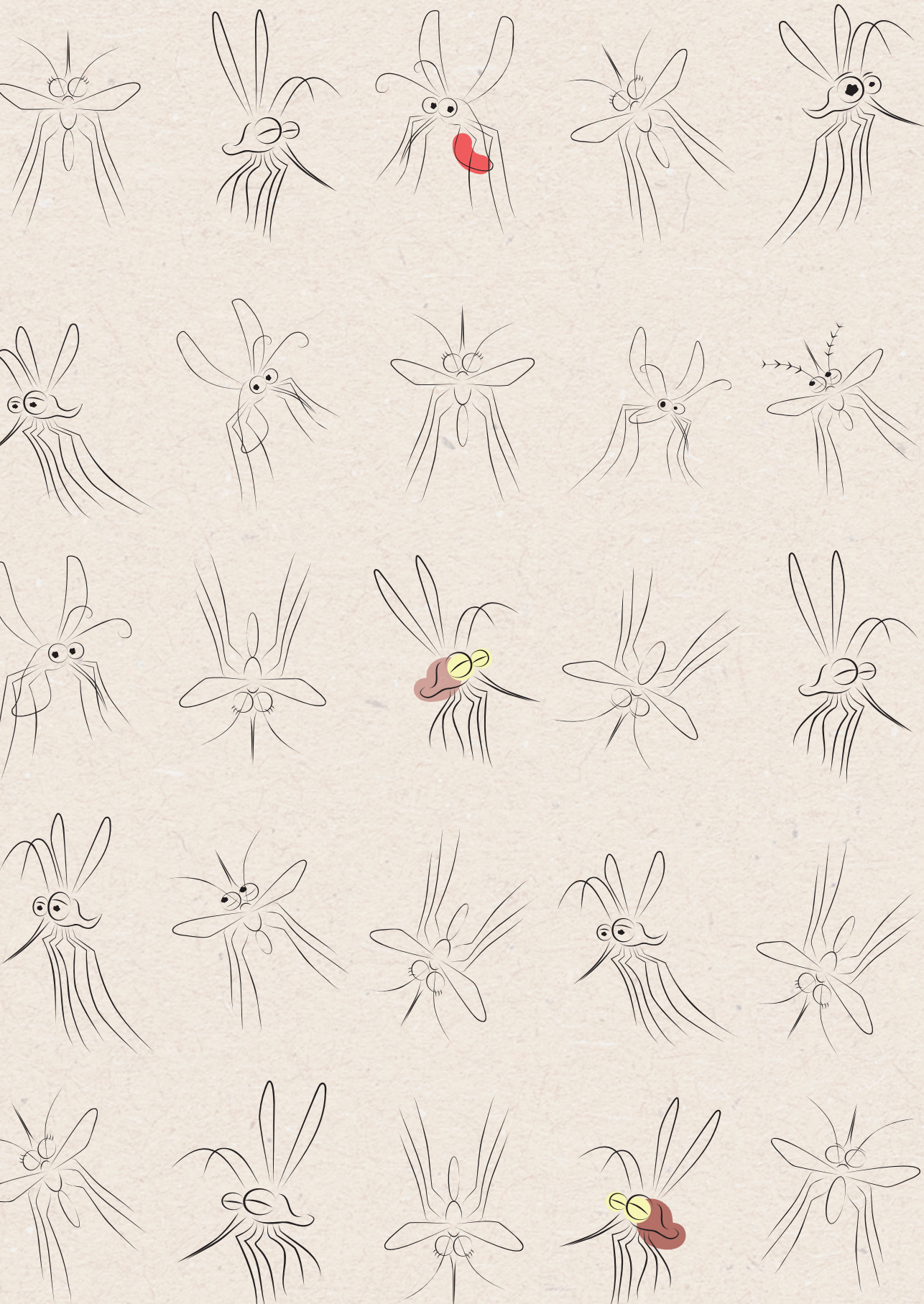
S4 Fig. Correlation between *Plasmodium* and viral infection levels. Each datapoint represents one virus and *Plasmodium* coinfecting mosquito, with the *Plasmodium* (COX1) Ct value on the x-axis and corresponding viral Ct value on the y-axis for (A) ASALV, (B) HEBV, (C) GOLV and (D) IIV6. Linear regression lines were fitted, and Pearson R^2 and P values are indicated.

Supplementary table S5. Primer sequences used in this study.

Description	Forward primer	Reverse primer
cell fusing agent virus	3'-GCAGCGGCGCTTTTGTGTGG- ⁵	3'-GCACTGCAAGGCATCCTCAC- ⁵
Kamiti river virus	3'-ATCCACAGCTGTAGGCGTTG- ⁵	3'-CAACCCGTCGTTTGGTTTC- ⁵
Culex Y virus	3'-GCACTACTTGAGCACTCGGT- ⁵	3'-GGTAGTATTGTCCGGCCCC- ⁵
cricket paralysis virus	3'-ACGAGGAAGCAACTCAAGGA- ⁵	3'-GAGCCGCTGAGATGTAAAG- ⁵
Flock House virus	3'-GTTTAATCGGACCGAAGTGC- ⁵	3'-TATTCCGGCTTCTACGTTGG- ⁵
invertebrate iridescent virus 6	3'-ACACCTATCATGGGAACTTGGG- ⁵	3'-GGACCCGTTTACCCTTCATTGTACG- ⁵
Ferak orthoferavirus	3'-GCATTGTTTAGCCTGTGTGCT- ⁵	3'-ATCAACCCTGTGTGGGTGAG- ⁵
Jonchet orthojoovirus	3'-AAAAGCGACACTAGCGGTTCT- ⁵	3'-AAGGGCTTTCATTCCCAACC- ⁵
Gouléako goukovirus	3'-AGCCCAAAACAAGTAGGGAG- ⁵	3'-CCTCCAGGAGCAAGATTGG- ⁵
Herbert herbevirus	3'-CGTGATTGTGATGCACAGGT- ⁵	3'-GGCCAGCTAAAGTCAACCTT- ⁵
Wallerfield virus	3'-ATTTGGGAGTGCCAGATTAC- ⁵	3'-ATACGTAGCGGCACCTTCCTG- ⁵
Piura virus	3'-TTTGTTCAACGCACACGTTTC- ⁵	3'-ACCACAGCCATCAGGAACAG- ⁵
goutanap virus	3'-TTCGTATACCTCGGGGTACC- ⁵	3'-TTGCAGCACTTCCAGATTCC- ⁵
Agua Salud virus	3'-CTCGAAGAGAGGCTCAACCAC- ⁵	3'-CGCAGAAGTAAGCGGTTTG- ⁵
Cavally virus	3'-CAGCAATACCATTTGCGGAC- ⁵	3'-TCCATGTCGGAGTAGTGCTG- ⁵
Otulum virus	3'-GATGCCAACGTAACCTAGC- ⁵	3'-ATGTGCCAAAAATCGTAAGCG- ⁵
Panama sandfly flavivirus	3'-CATGATGACCGCTTTGCTG- ⁵	3'-TGGTTCGAGCAGAAAGGGAAC- ⁵
Agua Salud negevirus	3'-TAACACGTGCTTTTGTGCGG- ⁵	3'-TGACCGGATCGGGTAAAAAC- ⁵

Supplementary table S5. Continued

Description	Forward primer	Reverse primer
<i>Anopheles</i> RPL5	3'-TCGGCCTGCACGTGCGCAACTACA-5'	3'-GGGATCCTGATGGAGGCGTGGGCG-5'
<i>An. gambiae</i> Cecropin 1	3'-TCCAAGATCTTCATCTTTGTGG -5'	3'-CTTAACGGCTGCCACCAC-5'
<i>An. gambiae</i> Defensin 1	3'-TACCCCTTCTGGAGGAAGTGC -5'	3'-CACACACCGCCCTTACTGTTG -5'
<i>An. gambiae</i> Gambicin 1	3'-TGAAAGCAGGTGTGCATTCTT-5'	3'-AACTTTTCGCTTGCAGTCCTC-5'
<i>Plasmodium</i> 18S rRNA	3'-TCGGATAACGAACGAGATCTTAAAC-5'	3'-ATGTATAGTTACCTATGTTCAATTCA-5'
<i>Plasmodium</i> COX1	3'-CATCAGGAATGTTATTGCTAACAC-5'	3'-CGATCTCCTGCATAATGTTGGGTC-5'



CHAPTER 6

The Hsf1-sHsp cascade has pan-antiviral activity in mosquito cells

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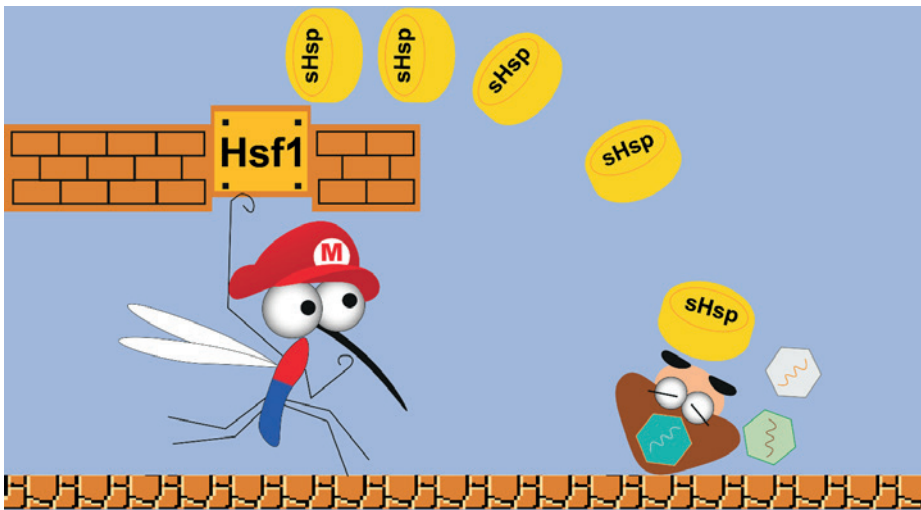
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Abstract

Aedes mosquitoes transmit pathogenic arthropod-borne (arbo) viruses, putting nearly half the world's population at risk. Blocking virus replication in mosquitoes is a promising approach to prevent arbovirus transmission, the development of which requires in-depth knowledge of virus-host interactions and mosquito immunity. By integrating multi-omics data, we find that heat shock factor 1 (Hsf1) regulates eight small heat shock protein (sHsp) genes within one topologically associated domain in the genome of the *Aedes aegypti* mosquito. This Hsf1-sHsp cascade acts as an early response against chikungunya virus infection and shows pan-antiviral activity against chikungunya, Sindbis, and dengue virus as well as the insect-specific Agua Salud alphavirus in *Ae. aegypti* cells and against chikungunya virus and O'nyong-nyong virus in *Aedes albopictus* and *Anopheles gambiae* cells, respectively. Our comprehensive *in vitro* data suggest that Hsf1 could serve as a promising target for the development of novel intervention strategies to limit arbovirus transmission by mosquitoes.



Introduction

Aedes mosquitoes are the primary vectors of arthropod-borne (arbo) viruses [1] that pose significant health and economic burden to humans [2-4]. Dengue virus (DENV) alone causes approximately 400 million infections and 20,000 deaths annually [5]. Outbreaks of chikungunya virus (CHIKV) in Europe and the Americas have also garnered significant attention [6-11]. With the continued resurgence of arboviruses and their unprecedented geographical spread over the past years, there is a pressing need for novel strategies to prevent and mitigate future outbreaks.

Arbovirus transmission occurs primarily through a horizontal cycle between blood-feeding arthropod vectors, such as mosquitoes, and vertebrate hosts. Female mosquitoes ingest viruses during a blood meal from an infected host and then transfer viruses into a naïve host through biting [12, 13]. Blocking virus transmission at the mosquito stage, rather than solely targeting the virus in human hosts, constitutes a promising, alternative approach to prevent arbovirus spread [14]. The development of such approaches requires in-depth knowledge of mosquito antiviral immunity.

Our current understanding of the insect immune system is largely informed by studies in *Drosophila melanogaster* [15-18], where the RNA interference (RNAi) pathway serves as a major antiviral response that degrades viral RNA and inhibits replication [19-21]. Innate immune signaling pathways, specifically the NF- κ B (Toll and IMD) and JAK-STAT pathways, have also been implicated in antiviral defense [16, 22]. However, *Drosophila* does not recapitulate mosquito specific processes such as virus acquisition and transmission via blood-feeding [12, 13, 15, 22]. It is therefore crucial to study immunity against arboviruses in relevant species, such as *Ae. aegypti*, the primary vector of the urban virus transmission cycle [23, 24].

RNAi has been identified as a major antiviral response in *Ae. aegypti* mosquitoes, for example, against CHIKV [25], Zika virus (ZIKV) [26, 27], DENV [28, 29], and Sindbis virus (SINV) [30]. In contrast, the role of classical innate immune pathways is less well-defined and seems to be virus dependent [31-36]. For instance, ZIKV infection activates the Toll pathway, while DENV infection activates both the Toll and JAK-STAT pathways [31-33]. Activation of the Toll and JAK-STAT pathways by depleting pathway-specific negative regulators Cactus and PIAS, respectively, suppresses ZIKV infection [31]. On the other hand, activation of the IMD pathway by silencing of Caspar did not affect ZIKV infection [31], but impaired SINV replication [34]. Additionally, stimulation with either heat inactivated *Escherichia coli* to activate the JAK-STAT and IMD pathways or *Bacillus subtilis* to activate Toll pathway had no

effect on CHIKV replication [25], suggesting unknown antiviral mechanisms yet to be discovered.

To investigate the transcriptional responses of *Ae. aegypti* to viral infection, we conducted a time-course RNA-seq experiment upon CHIKV infection. Our combined analyses of RNA-seq and chromatin profiling data revealed an early-responsive cascade, consisting of heat shock factor 1 (Hsf1) driving the expression of eight small heat shock proteins (sHsps). This Hsf1-sHsp cascade displayed conserved pan-antiviral activities in *in vitro* experiments on three vector mosquitoes. As such, our data suggest that Hsf1 serves as a promising target for the development of new intervention strategies to control arbovirus transmission by mosquitoes.

Results

The Hsf1-sHsp cascade acts as an early response to CHIKV infection

To uncover novel components involved in antiviral defense in *Aedes* mosquitoes, we performed a time-course RNA-seq experiment to determine differentially expressed genes (DEgenes) upon CHIKV infection of *Ae. aegypti* Aag2 cells at 4-, 8-, and 48-hours post-infection (hpi) (Fig. 1A and Supplementary Data 1). The CHIKV strain that emerged during the 2005–2006 outbreaks, which acquired a mutation (A226V) in the E1 envelope glycoprotein, was used in this study [37]. A multiplicity of infection (MOI) of 5 was used to ensure that the vast majority of cells are synchronously infected. Pilot RT-qPCR indicated that CHIKV RNA levels strongly increased from 4 hpi onwards (Supplementary Fig. 1A), mirroring the dynamics of CHIKV RNA reads in our RNA-seq data (Supplementary Fig. 1B). At 4 hpi, only a single DEgene was detected (*AAELO00080*, encoding a putative phosphoenolpyruvate carboxykinase), which was downregulated at 8 hpi (Fig. 1A). We observed major transcriptional changes at 8 hpi and 48 hpi, which we termed the early and late responses to CHIKV infection, respectively. The late response was more pronounced, with eight upregulated and 51 downregulated genes (Fig. 1A), and KEGG pathway analysis suggested that downregulated DEgenes were mainly involved in glycolysis, which we have reported before [38]. In contrast, the early response was modest, with two downregulated and 19 upregulated genes, which were enriched for genes encoding HSP20-like chaperones (Fig. 1A). Protein domain analysis revealed four genes encoding heat shock protein 70 (Hsp70) and eight genes encoding small heat shock proteins (sHsps) (Fig. 1B), which function to prevent protein aggregation and help cells to survive stressful conditions [39].

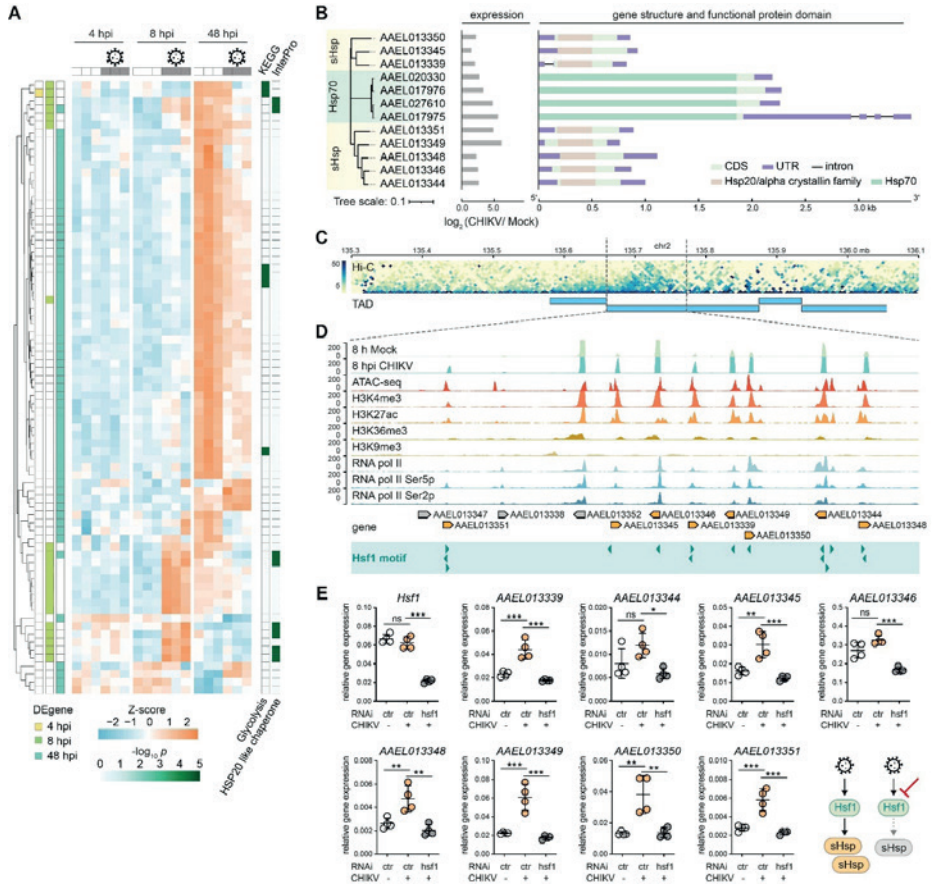


Figure 1. Hsf1 activates sHsp expression during the early stage of CHIKV infection. (A) Hierarchical clustering of differentially expressed (DE) genes at 4, 8, and 48 hpi of mock or CHIKV infection (MOI=5) in *Aedes aegypti* Aag2 cells, as quantified from RNA-seq data ($n=3$ biological replicates). DE genes ($p < 0.05$) at each time point are color labeled on the left; KEGG pathway and InterPro enrichment is shown on the right. Z scores were calculated based on log-transformed Fragments per Kilobase per Million mapped reads (FPKM). (B) Gene structure and protein domain analysis of heat shock proteins upregulated at 8 hpi of CHIKV infection. A neighbor-joining tree was constructed based on amino acid sequences (left). The $\log_2$ fold changes in gene expression were quantified by RNA-seq (middle). The gene structure and InterPro protein domains are shown along the gene body (right). (C) Hi-C interaction track with topologically associated domains (TADs) shown as horizontal blue bars ($p < 0.05$). (D) Genome browser tracks showing RNA-seq data of mock and CHIKV infected Aag2 cells and chromatin profiling data from uninfected Aag2 cells (ATAC-seq, histone marks and RNA pol II ChIP-seq) within the indicated TAD. Eight upregulated sHsp genes at 8 hpi are highlighted in orange, other genes in gray. Predicted Hsf1 motifs are shown as green arrowheads at the bottom. (E) Expression of the indicated genes in mock (-) or CHIKV (MOI=5) infected Aag2 cells upon RNAi-mediated silencing of Hsf1 at 8 hpi. Luciferase dsRNA served as a non-targeting control (ctr). RT-qPCR was performed in biological quintuplicates and shown as dots, with horizontal lines and the bars indicating mean $\pm$ SD. Symbols denote statistical significance in unpaired t -tests (ns, not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

While Hsp70 is involved in distinct steps of the viral cycle of dengue and Zika virus in mosquito cells, including entry, RNA replication, and virion assembly [40, 41], the role of sHsps during virus infection is largely unknown [42, 43]. Inspecting the genomic distribution of these sHsp genes, we found that all eight sHsps are encoded within one Topologically Associated Domain (TAD) deduced from Hi-C seq data [44] (Fig. 1C), suggesting that these sHsp genes are likely to be coordinately regulated by the same cis-regulatory network [45, 46]. To further study the regulation of sHsp gene expression within this TAD, we mapped our chromatin profiling data from Aag2 cells [47], and performed motif scan within open chromatin regions defined by the Assay for Transposase-Accessible Chromatin with sequencing (ATAC-seq). We found the DNA binding motif of the transcription factor heat shock factor 1 (Hsf1) to be highly enriched at promoter regions of these eight sHsp genes, but not at other genes within the same TAD (Fig. 1D). These regions were also marked by active histone marks H3K4me3 and H3K27ac, as well as RNA polymerase II occupancy, indicating active transcription [48, 49]. These findings together suggest that Hsf1 is the transcription factor that regulates sHsp gene expression. To experimentally test this hypothesis, we RNAi silenced *Hsf1*, and indeed, the induction of all eight *sHsps* upon CHIKV infection was significantly diminished (Fig. 1E). Hence, the Hsf1-sHsp cascade functions as an early transcriptional response to CHIKV infection in Aag2 cells.

The Hsf1-sHsp cascade displays anti-CHIKV activity

To explore the antiviral potential of the identified Hsf1-sHsp axis, we silenced *Hsf1* using RNAi and observed elevated CHIKV RNA levels at 8 hpi (Fig. 2A). sHsp genes share high similarity at the nucleotide level, we therefore used two sets of dsRNA targeting the conserved Hsp20 domain to knock down all eight *sHsp* genes and observed significantly increased CHIKV RNA copies at 8 hpi (Fig. 2B). In contrast, knockdown of *Hsp70* did not affect CHIKV replication (Fig. 2C), although we cannot exclude that Hsp70 may affect other stages of the viral life cycle [40, 41]. These results collectively suggest that the Hsf1-sHsp axis has antiviral activity against CHIKV infection.

We then modulated the activity of Hsf1 with either the small molecule inhibitor KRIBB11 [50] or the activator hsf1 [51] (Fig. 3A-B). KRIBB11 inhibits Hsf1-dependent recruitment of positive Transcription Elongation Factor b to the promoters of target genes, thereby blocking gene activation [50]. KRIBB11 treatment significantly downregulated most *sHsp* genes after 12 h, although this effect got weaker after 24 h. The precise mechanism by which hsf1 activates Hsf1 remains unclear [51]. We observed strong activation of *sHsp* genes following hsf1 treatment at both 12 h and 24 h. To further validate our findings, we included another compound, Direct Targeted Hsf1 Inhibitor (DTHIB), reported as a direct human Hsf1 inhibitor by stimu-

lating the degradation of nuclear Hsf1 [52]. Unexpectedly, DTHIB treatment activated *sHsp* expression in Aag2 cells at both 12 h and 24 h, similar to *hsfa1* (Fig. 3A-B). Thus, DTHIB acts as an Hsf1 activator rather than an inhibitor in mosquito cells.

Subsequently, we assessed the impact of Hsf1 modulation on CHIKV infection using the aforementioned compounds (Fig. 3C-D). At 12 hpi, KRIBB11 treatment did not affect *Hsf1* expression, but led to more than 5-fold increase of viral RNA levels in Aag2 cells (Fig. 3C), a trend that remained concordant at 24 hpi (Fig. 3D). While the expression of most *sHsp* genes was significantly reduced with KRIBB11 treatment, counterintuitively, we observed de-repression or even activation of some *sHsps*, such as *AAELO13346*, *AAELO13349*, and *AAELO13351* at 12 hpi, which is likely a secondary effect of increased CHIKV replication (Fig. 3C). *Hsfa1* treatment significantly increased *Hsf1* expression and strongly activated all eight *sHsp* genes, leading to about a 25-fold reduction in CHIKV copies at 12 hpi and a 10-fold reduction at 24 hpi (Fig. 3C-D). In contrast, DTHIB did not affect *Hsf1* expression at 12 hpi, but still elevated *sHsp* genes and consequently repressed CHIKV replication (Fig. 3C). These findings align with our RNAi experiments, reinforcing the antiviral role of *sHsps*.

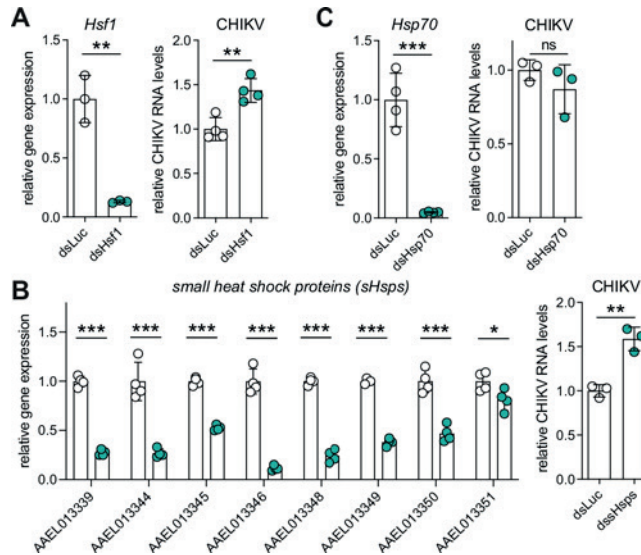


Figure 2. The Hsf1-sHsp axis has antiviral activity against CHIKV. (A–C) RNAi silencing of *Hsf1* (A) *sHsps* (B) *Hsp70* (*AAELO17975*) (C) and quantification of CHIKV RNA copies. dsRNA targeting the highly conserved Hsp20 domain was used to knock down all eight *sHsp* genes simultaneously in (B) RT-qPCR was used to assess target gene knockdown in uninfected Aag2 cells (left) and to quantify CHIKV RNA copies at 8 hpi (MOI=5) after target gene knockdown (right) in biological triplicate or quadruplicate, with luciferase dsRNA (dsLuc) serving as a non-targeting control. Individual data are shown as dots, with bars indicating mean ± SD. Symbols denote statistical significance in unpaired *t*-tests (ns, not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

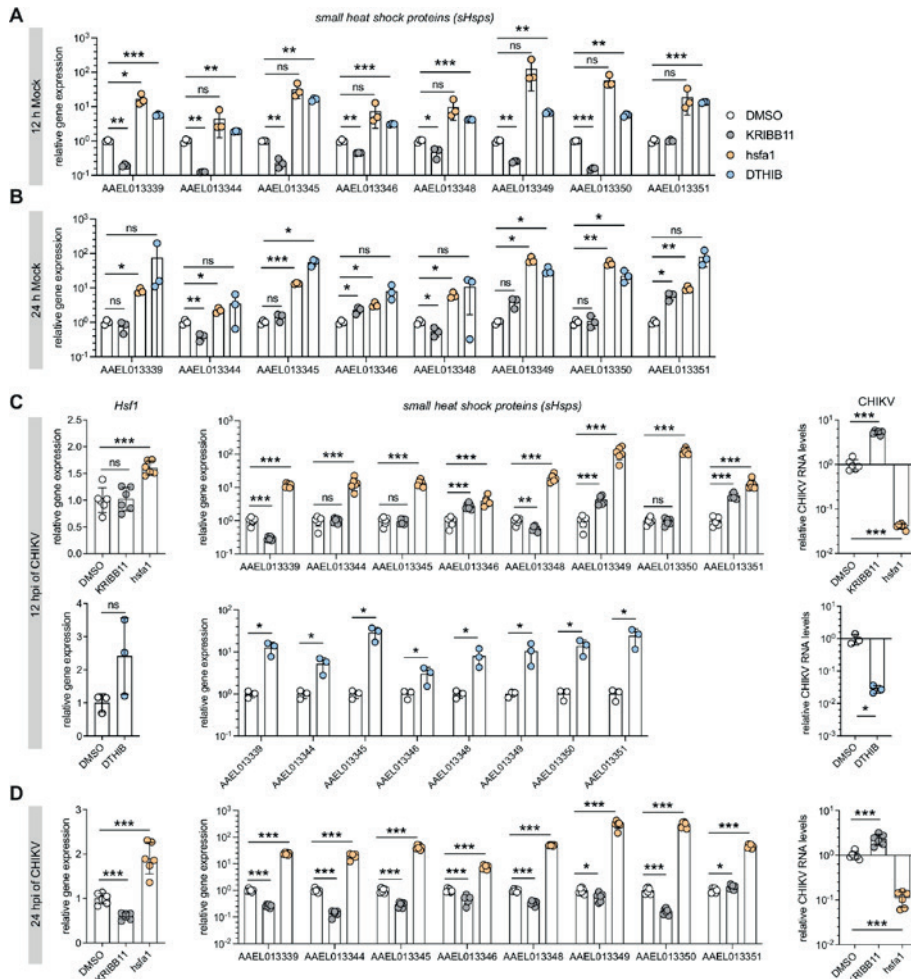


Figure 3. Pathway modulation using small-molecule compounds confirms the antiviral activity of the Hsf1-sHsps axis. (A, B) Effects of Hsf1 modulation using KRIBB11 (10 μ M), hsf1 (100 μ M), and DTHIB (10 μ M) on sHsp expression measured by RT-qPCR at 12 h (A) and 24 h (B). DMSO was used as control. (C, D) Expression analysis of *Hsf1*, sHsp genes, and CHIKV RNA copies following treatment with KRIBB11 (10 μ M), hsf1 (100 μ M), and DTHIB (10 μ M) at 12 hpi (C) and 24 hpi (D) with CHIKV (MOI=5), using DMSO as a control. RT-qPCR data are presented in biological triplicate, quadruplicate, or sextuplicate, displayed as dots, with bars indicating mean $\pm$ SD. Statistical significance in unpaired *t*-tests is denoted by symbols (ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001).

The Hsf1-sHsp cascade affects an early, post-entry stage of the viral replication cycle

To determine which stage of the CHIKV life cycle is affected by the Hsf1-sHsp cascade, we performed time-of-addition assays in Aag2 cells (Fig. 4A). DMSO treated cells were harvested at each indicated time point to establish a reference virus replication curve.

When the Hsf activator *hsfia* was added at time points before inoculation and up to 4 hpi, CHIKV replication was strongly inhibited (Fig. 4B). This observation suggests that *hsfa1* affects a critical step occurring around 4 hpi but does not impact earlier stages, such as the virus attachment or entry step, which typically occur within 1 hour after inoculation [53]. After 4 hpi, both positive and negative sense viral RNA copies became detectable, and the inhibitory effect of *hsfia* on CHIKV replication began to gradually diminish (Fig. 4C). This indicates that the Hsf1-sHsp cascade interacts with the early stage of CHIKV RNA replication but does not affect subsequent stages. To validate this time window, we performed a time-of-addition assay with the inhibitor KRIBB11 at 2, 4, 6, 8, 10 hpi, which showed the same pattern: a gradual loss of activation of CHIKV replication after 4 hpi (Fig. 4D-E). Together, these results demonstrate that the Hsf1-sHsp cascade likely reduces early CHIKV replication.

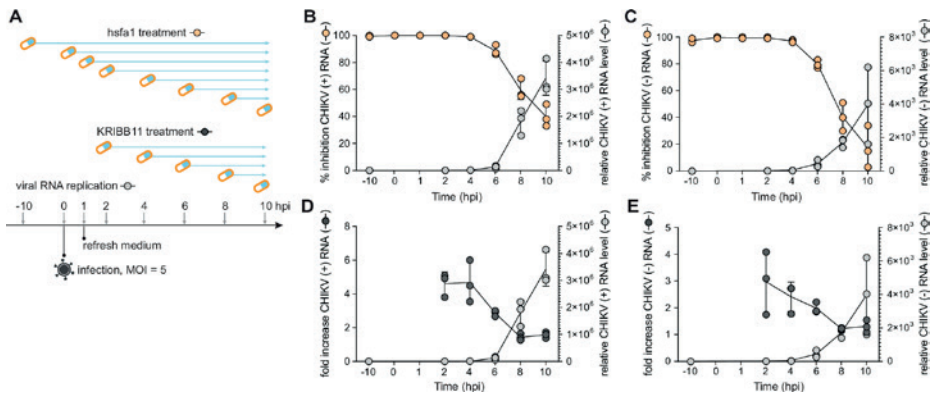


Figure 4. The Hsf1-sHsp cascade affects the early stage of the CHIKV replication cycle. (A) Schematic illustration of the time-of-addition assay during CHIKV infection (MOI=5) in Aag2 cells. Cell culture medium was refreshed to wash away unbound virus particles at 1 hpi. DMSO (light gray, Mock) was used to establish a virus replication curve, with samples harvested at the indicated time points. *hsfa1* (activator, orange) and KRIBB11 (inhibitor, dark gray) were added at specified time points, and cells were harvested at 10 hpi to assess their impact on CHIKV RNA levels. (B–E) The effect of *hsfa1* (100 μM) (B–C) and KRIBB11 (10 μM) (D–E) on CHIKV replication was assessed using RT-qPCR on positive sense (+) viral RNA (B,D) and negative sense (–) viral RNA (C,E). For comparative purposes, the replication curves of (+) and (–) viral RNA (light gray) have been duplicated in (B,D) and (C,E), respectively. Data are presented as mean ± SD and individual data of three replicates. DMSO was used as the negative control to calculate the percent inhibition upon *hsfa1* treatment B–C and the fold increase in CHIKV RNA levels upon KRIBB11 treatment (D–E).

The Hsf1-sHsp cascade showed pan-antiviral activity

We next investigated the effect of the Hsf1-sHsp cascade on replication of other alphaviruses in Aag2 cells, including SINV (AR339 strain), the insect-specific Agua Salud alphavirus (ASALV), and the flavivirus DENV (serotype 2). Consistently, KRIBB11 treatment led to increased virus replication for SINV and ASALV, whereas

hsfa1 treatment significantly reduced virus replication for SINV, ASALV and DENV (Fig. 5A-C). We extended our findings to *in vitro* experiments in other mosquito species and confirmed the antiviral property of the Hsf1-sHsp cascade in several natural virus-vector combinations [54, 55], such as CHIKV in *Aedes albopictus* U4.4 cells (Fig. 5D) and C6/36 cells (Fig. 5E), and O'nyong-nyong (ONNV) in *Anopheles gambiae* mos55 cells (Fig. 5F). The fact that the heat shock response was antiviral in both U4.4 cells and the RNAi-deficient C6/36 cells indicates that the Hsf1-sHsps cascade functions independently of RNAi. While the *in vivo* relevance remains to be determined (Supplementary Fig. 2), our results collectively suggest that the Hsf1-sHsp pathway displays a pan-antiviral role in cell lines from all three tested mosquito species.

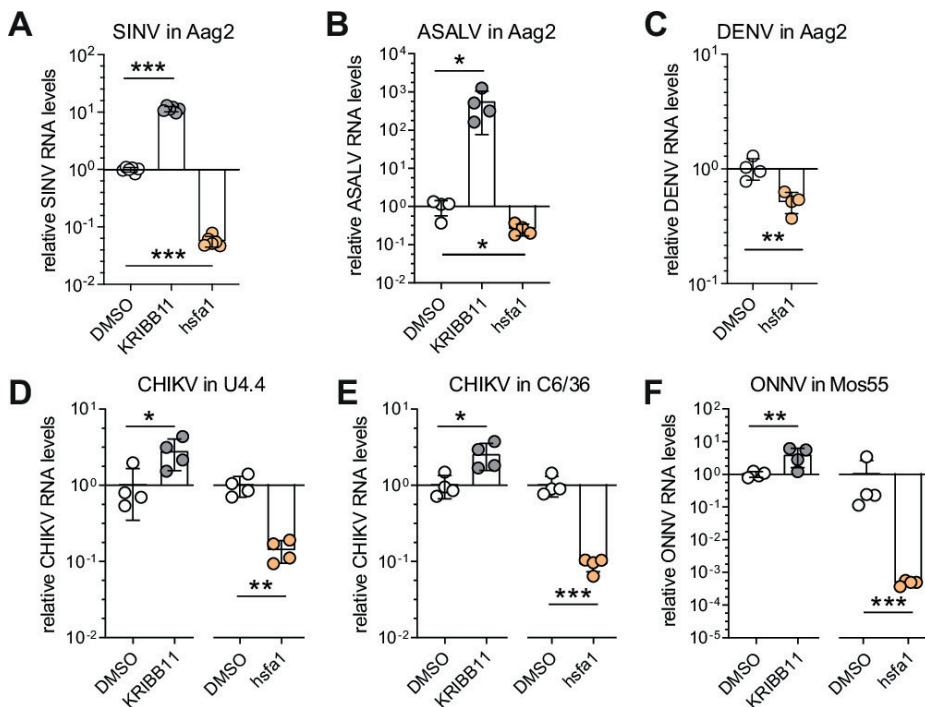


Figure 5. Pan-antiviral activity of the Hsf1-sHsp axis. (A–C) Viral RNA levels in *Aedes aegypti* Aag2 cells treated with KRIBB11 (10 μ M) or hsf1a1 (100 μ M) at 12 hpi of Sindbis virus (SINV, MOI=5) (A), at 12 hpi of Agua Salud alphavirus (ASALV, MOI=5) (B), and at 72 hpi of dengue virus (DENV, MOI=0.01) (C). (D–F) Viral RNA levels upon treatment with KRIBB11 (5 μ M) and hsf1a1 (100 μ M) at 12 hpi of CHIKV (MOI=5) in *Aedes albopictus* U4.4 cells (D), at 12 hpi of CHIKV (MOI=5) in *Dicer-2* deficient *Aedes albopictus* C6/36 cells (E), at 48 hpi of O'nyong-nyong virus (ONNV, MOI=0.5) in *Anopheles gambiae* mos55 cells (F). RT-qPCR was performed in biological quadruplicate or sextuplicate, shown as dots, with the bars indicating mean $\pm$ SD. Symbols denote statistical significance in unpaired *t*-tests (ns, not significant, **p* < 0.05; ***p* < 0.01; ****p* < 0.001).

Discussion

Our combined analysis of RNA-seq and epigenetic profiling data revealed an early-responsive Hsf1-sHsp cascade activated upon CHIKV infection, which we found to have broad *in vitro* antiviral activity against alphaviruses and a flavivirus in cell lines of three vector mosquito species: *Ae. aegypti*, *Ae. albopictus*, and *An. gambiae*. Overall, our work uncovers an antiviral cascade Hsf1-sHsp and identifies Hsf1 as a potential target for developing transmission-blocking strategies against arboviruses at the mosquito stage.

While RNAi is a well-established antiviral mechanism in mosquito vectors, it is becoming evident that inducible signaling pathways are of vital importance as well. In addition to the classical Toll, IMD, and JAK-STAT pathways, Wnt, mTOR, Ras/ERK, JNK pathways, and the Hsf1-sHsp cascade identified in this study, have been implicated in antiviral host defense [31, 33, 34, 36, 56-58]. Our time-course RNA-seq data revealed that the temporal activation of signaling pathways and associated genes is precisely regulated, with *sHsp* gene activation mainly restricted to the early stage of CHIKV infection and not detectable during later stages. In line with our findings, published RNA-seq data from an *in vivo* study of *Ae. aegypti* mosquitoes showed a similar pattern, with a higher induction of *sHsp* expression at the early stage (3 dpi) than at the late stage (7 dpi) of CHIKV infection [42]. In comparison to the well-studied role of Hsp70 at various stages of the viral cycle [40, 41, 59, 60], few studies analyzed the role of sHsps in viral infection. This gap in research may be due to the transient activation patterns of sHsp genes during the early stages of virus infection. Their activation is highly dependent on the virus infection dynamics, making it challenging to capture this fleeting time window. In addition, arboviruses need to overcome tissue barriers to establish a persistent infection of the mosquito. Consequently, the infection state of different tissues or body parts can vary significantly. This may explain why only two studies reported the activation of sHsps [42, 43], among the many transcriptome studies that have been conducted in the context of CHIKV infection in *Aedes* mosquitoes [36, 42, 43, 61-64]. Single-cell RNA-seq has emerged as a powerful tool that may help to address the heterogeneity of sHsp activation in a tissue-specific or even cell-specific context.

To gain deeper insights into the regulation of *sHsp* genes, we performed integrative analyses of 3D chromatin conformation (Hi-C seq) and chromatin profiling data (ATAC-seq and ChIP-seq) and identified Hsf1 as the upstream transcription factor that regulates expression of eight *sHsp* genes within one TAD. Targeting the transcription factor Hsf1, rather than individual downstream genes, is expected to be more effective for antiviral control. Together, our multi-omics analysis strategy

demonstrates its power in discovering novel antiviral signaling pathways, which can be applied to further investigate virus-vector interactions in different tissues, in other mosquito species, and in other arbovirus infections.

The mechanism underlying the antiviral activity of the Hsf1-sHsp cascade remains largely elusive. Our time-of-addition assays suggest that the response inhibits an early post-entry stage of the viral replication cycle. As Hsf1 activation led to a broad antiviral effect in three vector mosquitoes, it is likely that the Hsf1-sHsp cascade interacts, either directly or indirectly, with a common viral protein feature or a conserved host protein complex. It has been suggested that sHsps maintain protein homeostasis by binding proteins in non-native conformations, thereby preventing substrate aggregation [65]. The fact that a wide range of proteins is protected from precipitation suggests that sHsps may act rather promiscuously, similarly to the molecular chaperone Hsp70 [65-69]. It will be of great interest to profile sHsp associated proteins using proteomic approaches to identify sHsp client proteins and shed light on its antiviral mechanism.

To sum up, we identified the Hsf1-sHsp cascade as an antiviral mechanism in cell models of different vector mosquitoes. While the importance of the pathway for antiviral defense remains to be established *in vivo*, we propose Hsf1 as a potential target for developing arbovirus-blocking interventions. Considering the pan-antiviral activity of the Hsf1-sHsp cascade, our findings may open the way for the development of a broad-spectrum drug to prevent arbovirus transmission.

Materials and Methods

Cell lines

Ae. aegypti Aag2 cells were kindly provided by Raul Andino (University of California San Francisco), *Ae. albopictus* U4.4 cells by Gorben Pijlman (Wageningen University), and C6/36 cells by Sandra Junglen (Charité – Universitätsmedizin, Berlin). These cell lines were grown in Leibovitz's L-15 medium (Gibco 21083-027) supplemented with 10% fetal bovine serum (Sigma F7524-500ML), 50 U/ml Penicillin, 50 µg/mL Streptomycin (Gibco 15070-063), 1x non-essential Amino Acids (Gibco 11140-035), and 2% Tryptose phosphate broth solution (Sigma T8159-100ML). *An. gambiae* mos55 cells were kindly provided by Jason L. Rasgon (The Pennsylvania State University) and were grown in Schneider's insect medium (Gibco 21720024) supplemented with 10% fetal bovine serum, 50 U/ml Penicillin, and 50 µg/mL Streptomycin. Mosquito cell lines were cultured at 28°C without CO₂. BHK-15 (kind gift of Jolanda Smit, University of

Groningen) and BHK-21 cells (ATCC) were grown in Dulbecco's modified Eagle medium (Gibco 41965039), supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Mammalian cells were cultured at 37°C, with 5% CO₂.

Viruses

The CHIKV expression plasmid encoding the Leiden synthetic wildtype strain (LS3, GenBank: JX911334.1) was kindly provided by Dr. M.J. van Hemert (Leiden University Medical Center) and viral RNA was obtained by *in vitro* transcription on linearized plasmids using T7 mMessage mMachine (Invitrogen AM1344) [37]. RNA was then transfected into BHK-21 to grow the infectious virus. SINV AR339 strain was obtained by transfecting *in vitro* transcribed RNA from linearized plasmid pTE3' 2J in BHK-21 cells [70]. ASALV (GenBank: MK959114), kindly provided by Sandra Junglen (Charité, Berlin), was originally isolated from the homogenate of mosquito pool MP416 and grown in C6/36 cells [71]. ONNV plasmid was kindly provided by Prof. Dr. Andres Merits (University of Tartu), *in vitro* transcribed, and grown in C6/36 cells [72]. DENV serotype 2 (DENV2, strain 16681) was provided by Beate Kümmerer (University of Bonn) and stocks were prepared on C6/36 cells. For the quantification of viral stocks, CHIKV, SINV, and ONNV were titrated by end-point dilution on BHK-21 cells, DENV2 was titrated on BHK-15 cells, and ASALV was titrated on C6/36 cells. A day before the titration, 1x10⁴ mammalian or 5x10⁴ C6/36 cells were seeded per well in a 96-well flat bottom plate. A 10-fold serial dilution of virus samples was added to the cells in quadruplicate for the titration. After an incubation of 5 days, cells were inspected for cytopathic effect. The virus titer was calculated according to the Reed and Muench method [73].

Mosquitoes

Ae. aegypti mosquitoes (Black Eye Liverpool strain, obtained from BEI Resources, NR-48921), were reared at 28 °C, with 70% humidity and automated room lighting set at a 12:12 hours light/dark cycle. Larvae were fed with Tetramin Baby fish food (Tetra). Adult mosquitoes were fed with a 10% sucrose solution. Three-to-five-day-old female mosquitoes were used for experiments. For blood feeding experiments, mosquitoes were starved for 24 h prior to the experiment. CHIKV virus stock (5 × 10⁸ TCID₅₀/mL) and full human blood (Sanquin) was mixed at a 1:2 ratio to prepare the infectious blood meal with a final virus titer of 1.7 × 10⁸ TCID₅₀/ml. DMEM medium was mixed in the same ratio with human blood to prepare the mock inoculum. Blood feeding was performed with the Hemotek system for half an hour. Afterwards, mosquitoes were anesthetized on a CO₂ pad. Only blood fed mosquitoes were selected and kept in culture until the indicated time points. Mosquitoes were tissue dissected as previously described[74]. Dissected body parts or tissues were homogenized in either 200 µl DMEM medium using a Precellys 24 homogenizer program 5 (Bertin technologies).

RNAi in mosquito cells

PCR products flanked by T7 promoter sequences at both ends were *in vitro* transcribed by T7 polymerase to produce dsRNA using homemade T7 RNA polymerase. For the formation of double-stranded RNA, the reactions were heated to 90 °C for 10 minutes and then allowed to gradually cool to room temperature, and RNA was purified using the GenElute Mammalian Total RNA kit (Sigma RTN350) according to the manufacturer's protocol. Two sets of dsRNA targeting the Hsp20 domain were used to knock down *sHsp* expression (Fig. 2B) and two sets of dsRNA targeting the Hsp70 domain were used to knock down *Hsp70* (Fig. 2C). For detailed oligonucleotide information, see Supplementary Table 1.

Aag2 cells were seeded at a density of 1.5×10^5 cells/well in a 24-well plate, 24 h before the dsRNA transfection. For each target gene, a transfection mix containing 300 μ l non-supplemented L-15 medium, 500 ng dsRNA, and 1.3 μ l X-treme GENE HP DNA transfection reagent (Roche 6366546001) was prepared according to the manufacturer's recommendations. Per well, 100 μ l of the transfection mix was added in a dropwise manner. Cell culture medium was refreshed at 4 h post-transfection. Three days post-transfection, cells were either harvested for evaluation of target gene knockdown or infection with CHIKV at an MOI of 5.

RNA isolation and RT-qPCR

Aag2 cells were homogenized in RNA-Solv reagent (Omega Bio-Tek) and RNA extraction was performed according to the manufacturer's instructions. Briefly, 200 μ l of chloroform was added to 1 mL RNA-Solv reagent and thoroughly mixed. After centrifugation, the aqueous phase was collected, and RNA was precipitated using isopropanol. This mix was incubated for 1 hour at 4 °C, followed by centrifugation to pellet the RNA. Pellets were washed twice in 80% ethanol and dissolved in nuclease free water. RNA integrity was evaluated on ethidium bromide-stained agarose gels, and RNA concentration was determined using the Nanodrop ND-1000.

For RT-qPCR analyses, DNaseI (Ambion)-treated RNA was reverse transcribed with random hexamers using the TaqMan Reverse Transcription Reagents (Invitrogen N8080234) and PCR amplified using the GoTaq qPCR system (Promega A6002) on a LightCycler 480 (Roche). In RNAi experiments, expression levels of target genes were normalized to the expression of the housekeeping gene *lysosomal aspartic protease* (*LAP*, *AAEL006169*), and fold changes were calculated using the $2^{-\Delta\Delta CT}$ method [75]. All primers are listed in Supplementary Table 1.

RNA-seq library preparation and analysis

For RNA sequencing in Aag2 cells, control dsRNA (dsLuc) treated cells were infected with CHIKV at an MOI of 5. The dsRNA treatment was included in the procedure to allow a better comparison of the 4 hpi and 8 hpi samples to the 48 hpi datasets that was generated in parallel for a previously published study (PRJNA885496) [38].

TruSeq Stranded mRNA kit (Illumina 20020594) was used for library preparation from total RNA according to the manufacturer's protocol. The prepared libraries were quantified and controlled for sample quality using High Sensitivity DNA Chip on a DNA1000 Bioanalyzer (Agilent 5067-4626), and the libraries were analyzed on an Illumina HiSeq 4000 sequencer (GenomEast Platform).

After initial quality control by FastQC [76], raw sequence reads were aligned to the reference genome, *Ae. aegypti* LVP (VectorBase, release 56) and the plasmid CHIKV genome (LS3, GenBank: JX911334.1), using STAR [77] with default settings. Quantification of gene expression (FPKM) was performed with Cuffdiff [78]. R package DESeq2 was used for differential gene expression analysis (with adjusted p value < 0.05) [79]. Genes were considered expressed if the mean of the DESeq2-normalized counts (basement) was higher than 20. The R package pheatmap was used to generate the heatmap for DEgenes upon CHIKV infection, which was based on z-scores of normalized gene expressions ($\log_{10}$ FPKM) [80]. DEgenes are listed in Supplementary Data S1.

Bioinformatic analyses

STRING was used for KEGG pathway and InterPro protein domain enrichment analysis [81]. Detailed protein domain analysis was performed using InterPro [82].

For 3D chromatin conformation analysis, TADs were defined by analyzing Hi-C data (GSE95797) [44] with HiCEXplorer [83]. A cutoff of $p < 0.05$ was used, after correcting for multiple tests. pyGenomeTracks from the deepTools package [84] was used to visualize gene expression pattern, TADs, and previously published chromatin profiling data from Aag2 cells, including ATAC-seq, histone mark ChIP-seq and RNA pol II ChIP-seq (GSE210270) [47]. All genome browser tracks were normalized to FPKM (y-axis). Open chromatin regions were defined as ATAC-seq peaks called by MACS2 with standard settings and a q value of 0.05 [85]. Homer was used for DNA motif scan on ATAC-seq peaks [86].

Compound experiment

KRIBB11, Hsf1a, and DTHIB were purchased from Axon Medchem (catalog numbers 2538, 1890, and 3412, respectively). All compounds were dissolved in DMSO to

a final concentration of 10 mM (KRIBB11) or 100 mM (Hsf1a and DTHIB) and stored at 4°C for use within one month. Mosquito cells were seeded at a density of 1.5×10^5 cells/well in a 24-well plate. Cells at 50% confluency were treated with the indicated concentrations of compound or an equivalent volume of DMSO and immediately afterwards inoculated with virus at the indicated MOI. Cell lysates were collected at the indicated time points for RNA isolation and RT-qPCR. For the time-of-addition assay, the same procedures were followed, except that the medium was replaced at 1 hpi. For mosquito experiments, DTHIB was directly added to the (infectious) blood meal at a concentration of 10 μ M and mixed well before blood feeding, with DMSO serving as a control.

Statistics and reproducibility

RT-qPCR experiments in Fig. 1–5 were performed in biological triplicate, quintuplicate, sextuplicate, or octuplicate, with individual data shown as dot and the bars indicating mean $\pm$ SD. Statistical differences were examined by unpaired two-tailed *t*-tests. Nonparametric Mann-Whitney U and chi-squared tests were used for comparison of virus titers and infection rates, respectively, in Supplementary Fig. 2. All statistical tests were performed using GraphPad Prism 9.

Data availability

RNA-seq data have been deposited in NCBI Sequence Read Archive under accession number PRJNA885496. ATAC-seq, ChIP-seq data, and Hi-C data were available from previous studies, deposited under Gene Expression Omnibus accession number GSE210270 (ATAC-seq and ChIP-seq) and GSE95797 (Hi-C). Source data for other graphs in the main and supplementary figures are provided in Supplementary Data 2.

Authors' contribution

SRM performed the RNA sequencing experiment for a related study. JQ conducted the formal analysis of the sequencing data and identified the small heat shock proteins. JQ conceptualized the study, initiated the investigation and performed the first *in vitro* experiments, with support from MS and LC. JQ and MS jointly conducted the initial *in vivo* experiments. JQ wrote the original draft of the manuscript. For the revised version, MS performed some additional RNA sequencing analyses, *in vitro* and *in vivo* experiments and contributed by rewriting, editing and refining the final manuscript text. All authors reviewed and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

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Supplementary Materials

All supplementary materials can be downloaded from
<https://www.nature.com/articles/s42003-024-07435-4#additional-information>

Supplementary Table 1. Primers used in this study.

Supplementary Table 2. Differentially expressed genes (DEgenes) and FPKM values upon CHIKV infection of Aag2 cells at different time points.

Supplementary Data 1. Source data for the graphs in the paper.



CHAPTER 7

General Discussion

Plasmodium falciparum, the malaria parasite transmitted by *Anopheles* mosquitoes, causes more than 240 million cases of clinical malaria and around 600,000 deaths annually [1, 2]. The deployment of insecticides, chemicals to kill insects such as organochlorines like DDT and pyrethroids, in combination with broader access to effective antimalarial drugs have successfully reduced malaria incidence [3]. However, the increasing threat of insecticide resistance of vector mosquitoes and drug-resistance of the malaria parasite makes these approaches less effective [4], necessitating newly approved methods for control.

Novel malaria control strategies; Utilizing insect viruses in the fight against malaria

Mosquito vectors are the definitive hosts of *Plasmodium* parasites, where sexual reproduction takes place. They become infected after feeding on an infected human and, following completion of the extrinsic incubation period, can infect the next human host. This process makes mosquitoes critical targets for malaria interventions, and interrupting transmission can form a key strategy in reducing the malaria burden. One promising approach to achieve this is the use of a symbiont that prevents parasite development in the mosquito and thereby blocks transmission to humans. This symbiont may be an organism naturally coexisting with *Anopheles* mosquitoes. For example, the bacterium *Wickerhamomyces anomalus* secretes a killer toxin reducing *Plasmodium berghei* development in the midgut of *Anopheles stephensi* by approximately 65%, and it already occurs naturally in mosquito populations [5, 6]. Another approach can be the use of genetically modified symbionts, such as *Serratia* or *Pantoea agglomerans* bacteria engineered to express antimalarial effector proteins in the mosquito midgut, thereby affecting parasite fertilization in this environment, and ultimately reducing *Plasmodium* oocyst development [7, 8]. While these examples involve bacteria, other symbionts, such as insect specific viruses (ISVs), may also form a viable avenue to interfere with parasite development. This thesis investigates the use of ISVs as *Plasmodium*-reducing viral symbionts in *Anopheles* mosquitoes.

Chapter 1 starts by outlining potential targets that ISVs could manipulate to achieve transmission-reduction by examining which immune pathways are activated by malaria parasites. In *Chapter 2*, we review the known reservoir of ISVs in mosquitoes in an extensive literature study. *Chapter 3* presents data from a study where we performed small RNA sequencing on *Anopheles* mosquitoes from Burkina Faso with the aim to discover naturally occurring (new) ISVs. To explore and test candidate ISVs, we developed methods for *in vivo Plasmodium*-ISV co-infections in *Anopheles* mosquitoes,

that are described in *Chapter 4*. These co-infection methods are used in *Chapter 5*, where a panel of known ISVs is screened for their ability to interfere with *Plasmodium* development. *Chapter 6* explores the role of heat shock proteins in the mosquito antiviral response, highlighting a conserved Hsf1-sHsp cascade with broad antiviral activity.

Immune pathway modulation to block transmission; Hacking the mosquito's defenses

Plasmodium infection in mosquitoes triggers diverse innate immune pathways. Beginning at the midgut invasion, the presence of parasites activates strong innate, non-adaptive humoral and cellular immune responses, including Reactive Oxygen Species (ROS) production and insulin signalling [9-11]. Subsequently, ookinete traversal of the midgut epithelium triggers complement activation and melanization, that are crucial for eliminating malaria parasites [9-11]. The Toll, IMD, and JAK-STAT signalling pathways, which play key roles in regulating antimicrobial peptide production and immune effector expression in *Anopheles* mosquitoes, are also activated in response to *Plasmodium* infection [9-12]. In *Chapter 6* we identify the Hsf1-sHsp cascade as an early antiviral response in mosquitoes, thereby broadening our understanding of these immune pathways. Although its role in anti-plasmodial defense is not investigated, this newly identified cascade may exert indirect effects on *Plasmodium*. Collectively, these immune responses initiate the expression of a range of anti-infection factors, thereby shaping mosquito immunity that, along with other pathogens, target *Plasmodium* parasites. Although direct evidence for ISV-*Plasmodium* interactions is currently lacking, their co-occurrence may modulate shared immune pathways, such as Toll, IMD, and Jak-STAT, with potential effects on parasite development. In addition to the potential direct effects of ISVs on mosquito immune responses, it is critical to assess whether they can indirectly reduce *Plasmodium* sporozoite burden. This includes ISV-triggered immune activation that does not specifically target *Plasmodium* but may still lower transmission potential. Such indirect effects could occur when ISV infection stimulates broad acting immune- or stress-related pathways, creating a heightened immune state with a less favorable environment for *Plasmodium* development. This approach is especially promising because it may reduce malaria transmission without directly targeting the parasite, thereby avoiding strong evolutionary pressures that could lead to resistance. However, for such a strategy to be viable, the ISV must reduce sporozoite load enough to impact transmission, while not causing major fitness costs to the mosquito. Beyond individual-level effects, a thorough evaluation of ISV dynamics at the population level, and potential impacts beyond the targeted mosquito species, is essential.

In *Chapter 5*, we investigated whether ISVs affect *Plasmodium* development by quantifying sporozoite load in co-infected *Anopheles* mosquitoes using RT-qPCR. Sporozoite load was selected as the primary outcome measure because it reflects the final developmental stage of the parasite in the mosquito and is directly linked to potential infectiousness. Moreover, sporozoite density has been shown to influence the likelihood and intensity of onward transmission to vertebrate hosts, highlighting its significance as a biological indicator of transmission potential [13, 14]. Using RT-qPCR as the readout was technically advantageous as this allowed us to determine sporozoite and viral load within one analysis flow. However, earlier developmental stages, such as oocyst presence, could also be used as outcome measure, as it can be detected earlier in the infection process. This measurement is a more common practice in malaria research as it is safer to assess, as mosquitoes are not yet infectious. Our approach is also not without limitations, since only a fraction of sporozoites emerging from oocysts successfully reach the salivary glands. In fact, when released from the oocysts, only ~19% of all sporozoites reach the salivary glands and have a chance of subsequently infecting a mammalian host [15]. Even once the salivary gland is colonized, sporozoite load still does not guarantee transmission, as 39% of *Plasmodium falciparum* positive mosquitoes in Burkina Faso and 22% of *Plasmodium Yoelii* infected mosquitoes failed to expel sporozoites upon probing [14, 16]. However, only a few sporozoites need to be expelled to subsequently infect a mammalian host. For example, *Plasmodium berghei* infection in mice after a mosquito bite can already be established by a very low number of sporozoites deposited (ranging from 6 to 11) [17]. Despite sporozoite loss during travel to the salivary glands, successful invasion still occurs, making the mosquito infectious for its next human host. Nonetheless, it remains unclear whether ISV co-infection can interfere with this late-stage transmission process. Viral infections may activate immune pathways or compete for cellular resources in the salivary glands, potentially affecting sporozoite survival or invasion. Further studies should therefore explore whether ISVs can modulate *Plasmodium* transmission at the salivary gland level. Given the limitations of oocyst sporozoite load as a proxy for infectiousness, a more predictive measurement in *Chapter 5* would have been the actual number of sporozoites within expelled saliva. This can be assessed using a forced salivation assay, where saliva is expelled in sugar water [18], or, even more accurately, by quantifying sporozoites expelled into artificial skin during probing [14]. Even though measuring expelled sporozoites is more precise, sporozoite load is known to correlate strongly with transmission potential, making it unlikely that employing these alternative methods would have substantially altered the observed outcomes.

Effects of ISVs on mosquito survival and behaviour; ISVs beyond immunity

The effects of ISVs on mosquito behavior and mortality remain relatively understudied. In contrast, for several arboviruses, it is well established that systemic infection can alter mosquito physiology and behavior. For example, infections with dengue and Zika viruses have been shown to increase locomotor activity and blood-feeding propensity, likely through infection of the mosquito's central nervous system [19]. The model organism *Drosophila melanogaster* has been more extensively studied in the context of virus infections. For example, infection with Drosophila C virus leads to reduced survival, altered metabolism and notable behavioural changes such as decreased locomotion, increased sleep (in females) and changes in social spacing [20-24]. These detailed insights suggest that even mild or persistent viral infections can subtly but significantly alter insect physiology and behaviour, highlighting the potential for ISVs to have underappreciated impacts on mosquito ecology, behaviour, and possible vector competence. To date, no studies have investigated whether persistently infecting ISVs in mosquitoes can target the central nervous system or affect blood-feeding behavior in a manner analogous to arboviruses. From a vector control perspective, it is desirable that ISVs that reduce pathogen transmission do not cause substantial fitness costs to the mosquito, as this could limit their suitability as long-term intervention strategy. At the same time, if behavioral changes, such as increased biting frequency occur, they could pose a concern for vector-based malaria control strategies, as they may elevate malaria transmission risk in co-infected individuals. However, behavioral changes induced by ISVs could influence disease transmission in multiple directions. For instance, some alterations, such as reduced host-seeking activity or a shortened lifespan, could decrease mosquito-host contact and thereby even further reduce pathogen spread. From an evolutionary perspective, there may be little selective pressure for ISVs to induce such traits, as their transmission does not depend on vertebrate hosts. The often mild or asymptomatic effects observed for some ISVs may reflect long-term coevolution and host tolerance [25], though the possibility of subtle or unintended behavioral effects should not be excluded and warrants further investigation and long-term surveillance.

The prevalence and receptivity of our viral candidate within the population can be important for our desired outcome. Such information can provide insights into host population ecology, as development time and viability costs of the infected individual mosquitoes must be sustainable in terms of competition with uninfected populations. If development time or viability is negatively affected, and only a small portion of the population is infected, the trait might not be permanently established but outcompeted by uninfected individuals. In this case, a re-introduction or constant

exposure of our agent might be necessary to maintain a high prevalence of ISVs (and hopefully a related low *Plasmodium* infection burden in mosquitoes), which brings repeated interference of the ecosystem along. Regular assessment would be desirable to evaluate ISV prevalence and detect possible side effects. Mosquito population dynamics differ by season, and a seasonal introduction therefore might be favoured. Evidence of oral acquisition of the interfering virus must be experimentally validated to show the populations' infection dynamics and study feasibility.

Even for viral agents with a narrow host range, the possibility of infection of other mosquito species or closely related organisms in the same ecosystem must be tested. For example, *Wolbachia* in *Drosophila* confers resistance to *Drosophila C virus* [26] and in *Aedes aegypti* inhibits development of filarial nematodes [27] and reduces dengue and chikungunya virus loads [28]; yet it increases susceptibility of *Spodoptera* moths to nucleopolyhydrovirus [29] and has been suggested to enhance *Plasmodium* infection in *Anopheles* [30]. Unwanted ecological effects should be minimized through laboratory testing across species. Nevertheless, lab tests cannot guarantee absence of spillover in the field, thus small, controlled field trials and continuous monitoring of ecosystem stability are necessary.

From an evolutionary perspective, there is also a need to monitor for potential co-evolution between ISVs and *Plasmodium*. For instance, parasites may develop resistance to ISV-mediated interference, or ISVs could evolve to minimize their impact on *Plasmodium* if the interaction reduces their own fitness or transmission. These possibilities underscore the importance of long-term evolutionary surveillance.

Technological tools; Mining the mosquito virome

Historically, detection and identification of new viruses required labor-intensive methods, including cell culture and manual molecular assays. Since the rise of Next Generation Sequencing (NGS), virome studies have become more feasible because culture is no longer a prerequisite. Combined with declining costs, the number of studies in mosquito virology is increasing rapidly [31-33]. As more investigations are conducted each year, our understanding of the mosquito virosphere, its diversity, evolution, and ecology, grows substantially [34]. In *Chapter 2* we showed that the information about mosquito viromes grows fast, from just two publications per year between 2000 and 2008 to 27 studies published in 2021 alone, an increase of 1250% [34]. This increase is considerably more pronounced when compared to the

broader field of malaria research, which has a 189% publication increase during the same period, rising from 1906 publications in 2000 to 5508 in 2021 [35]. While the absolute number of mosquito virome studies remains smaller, the relative growth highlights a rapidly emerging research focus within vector biology.

Virome sequence data may be obtained via different library preparation techniques (e.g. total RNA or small RNA), sequencing platforms (e.g. Illumina or Oxford Nanopore), and sources (e.g. whole mosquito, larvae, eggs or specific tissues) as discussed in *Chapter 2*. Initial sample collection and processing methods also vary, from directly sampling environmental or wild specimens to employing prior culture enrichment. Whether sequencing is performed directly on the material or after culture enrichment, can cause distinct results as the latter approach can increase sensitivity by amplifying viral material but also can introduce bias by selectively favoring viruses that can be cultured. These methodological differences result in distinct requirements and considerations for reporting, interpreting, and utilizing virome data across studies. Minimal reporting standards, as proposed by Roux *et al.* 2018 [36] and discussed in *Chapter 2*, are essential to ensure data usability and comparability. Ideally, all mosquito virome data should be stored in freely accessible, user-friendly databases, either as a standalone resource, or integrated into existing platforms such as VEuPathDB (<https://veupathdb.org/>). However, maintaining and updating database resources incurs ongoing costs, and with the recent funding cuts, exclusive reliance on governmental support is no longer sustainable. Recent policy changes in the United States highlight this trend as more than 1,600 research grants, valued at US \$1.5 billion, were terminated under National Science Foundation and National Institutes of Health funded programs [37]. As a result, some data repositories sought alternative funding models, including subscription-based access. However, restricting access to paying users may disproportionately disadvantage researchers in low- and middle-income countries. Even if the development of such a database is technically feasible, ongoing personnel and institutional support will still be required to ensure continuous monitoring, curation, and updating of the resource.

As shown in *Chapter 2*, most mosquito virome studies focus on *Culex* (114 studies) and *Aedes* (85 studies), while only 34 studies reported insect viruses in *Anopheles* mosquitoes [34]. I consider it unreasonable to assume that there are simply fewer viruses present in the anopheline virosphere compared to other mosquito species. It is more likely that anopheline viromes are comparatively understudied, in part because *Anopheles* is less frequently associated with arbovirus transmission and research focusses more on *Plasmodium*. To expand our understanding, we conducted small RNA sequencing of *Anopheles gambiae* (*An. gambiae*) mosquitoes sampled in Burkina Faso in *Chapter 3*.

Table 1. Comparison of methodological approaches and outcomes between two reviews. Key differences include study scope, database selection, screening methods, and virus classification systems, which likely contributed to the observed discrepancies in the number and types of viruses reported.

Feature	Moonen, Schinkel, van der Most et al., 2023	Hernández-Valencia et al., 2023
Focus organism(s)	<i>Aedes, Anopheles, Culex</i>	<i>Anopheles</i>
Virus types	ISVs	Arboviruses, probable arboviruses, ISVs
Databases	PubMed	EBSCOhost, Google Scholar, ScienceDirect, Scopus, and PubMed
Studies including virome in <i>Anopheles</i> (n)	34	203
Screening method	Manual, full text and supplements	Unclear
Virus classification	NCBI taxonomy	ICTV taxonomy
Unique viruses reported in <i>Anopheles</i> (n)	174	161

Among the viruses identified in *Anopheles* mosquitoes, the majority belong to the *Flaviviridae* family, and were detected at comparable frequencies using both high-throughput sequencing and PCR-based methods. A systemic review by Hernández-Valencia *et al.*, 2023 reported 161 distinct viruses associated with anopheline mosquitoes across 203 studies, comprising 35 arboviruses, 24 probably arboviruses, and 84 putative ISVs. These findings differ notably from those presented in *Chapter 2*, that documents 174 unique viruses identified in *Anopheles* mosquitoes from only 34 studies (Table 1) [34, 38]. This discrepancy may be attributed to differences in search strategies and data extraction methods. Our analysis in *Chapter 2* was restricted to PubMed and employed a stringent, reproducible search protocol. In contrast, Hernández-Valencia *et al.* utilized a broader database search strategy, including EBSCOhost, Google Scholar, ScienceDirect, Scopus, and PubMed. Moreover, while Hernández-Valencia *et al.* describe data extraction from full articles in their methods section, the exact methodology remains unclear. In contrast, our approach involved manual review of all included studies, encompassing main text, figures, and supplementary materials, which likely allowed for the identification of a greater number of reported viruses, particularly ISVs that may not have been explicitly listed or indexed. Taxonomic classification criteria also differed between the two studies. *Chapter 2* employed the NCBI Taxonomy database to define unique viral entries, whereas Hernández-Valencia *et al.* relied on the International Committee on Taxonomy of Viruses (ICTV), which may exclude unclassified or newly identified ISVs. In addition, a key factor contributing to the discrepancy appears to be differences in study scope and inclusion criteria. Hernández-Valencia *et al.* included a wide range of virus types (arboviruses,

probable arboviruses, and ISVs), while *Chapter 2* places greater emphasis on ISVs confirmed through metagenomic detection. Furthermore, inconsistent virus reporting across primary studies, particularly where viruses are mentioned only in figures or supplementary data, may have contributed to underreporting in reviews. These observations underscore how incomplete or inconsistent reporting practices in virome studies, combined with differing inclusion criteria, can obscure true viral diversity and limit cross-study comparisons. This reinforces the need for standardized and transparent reporting protocols, as previously discussed in *Chapter 2* and revisited in *this chapter*, to ensure the accurate interpretation and comparability of virome datasets.

Limitations of current experimental models; Beyond the petri dish

Confirmation of the receptivity of *Anopheles* mosquitoes to ISVs can benefit from different experimental models. Several *An. gambiae* cell lines are available to this purpose; we extensively used the 4a-3B and mos55 lines in *Chapter 5*. Both lines are derived from larvae, are immortalized, and hemocyte derived, although they differ morphologically [39-41]. Viruses discussed in *Chapter 3 and 5* are known to infect the *An. gambiae* mosquitoes, yet their tissue tropism and associated immune activation remain poorly characterized both *in vitro* and *in vivo*. Insect viruses often exhibit distinct cell tropisms within their hosts, preferentially infecting specific tissues such as the midgut epithelium, salivary glands, or fat body [42, 43]. These tissue-specific interactions influence viral replication dynamics and shape the resulting immune responses. Since each mosquito cell type can respond differently to viral infection, *in vitro* studies using non-representative cell lines may identify immune responses and gene expression profiles that do not necessarily accurately reflect *in vivo* conditions. This may potentially limit the physiological relevance of experimental findings. In our *in vitro* experiments, we selected ISVs based on robust replication, but we have no evidence that our used lines are representable for the true target cells in mosquitoes. Other *An. gambiae* lines, such as Sua, Ag55 and Agmm [44], also exhibit a hemocyte-like gene expression profile and are therefore probably similar to the lines we used. Unfortunately, mosquito cell lines derived from a broader range of tissues are currently not available, highlighting the need for more diverse and physiologically relevant *in vitro* culture systems. Ideally, cell lines used for *in vitro* viral studies should not only permit efficient replication but also reproduce the immune signaling and cellular context of the natural target tissue. Ultimately, validating these models by correlating *in vitro* results with *in vivo* infection outcomes is essential to ensure that conclusions drawn from cell culture systems are biologically meaningful.

In *Chapter 4, 5 and 6* we therefore also use *in vivo* mosquito infections to complement our findings in cell cultures, aiming to provide a more complete and physiologically relevant model. Working with whole mosquitoes captures the complexity of tissue-specific viral replication, systemic immune responses, and potential effects of vector competence, factors that are often not present in cell culture models. All *in vivo* experiments in *Chapter 4 and 5* are conducted using the *Anopheles stephensi* mosquito, Sind-Kasur strain. This strain is a cross from a mosquito from the Sind and a mosquito from the Kasur province in Pakistan, and was originally selected for its high infection prevalence and limited intrapopulation variability [45]. Now maintained in the lab, this strain likely has adapted to laboratory conditions, with inbreeding and genetic drift possibly increasing genome-wide homozygosity, where individuals possess two identical copies of a DNA sequence [46]. One might question whether such lab-adapted mosquitoes accurately reflect natural populations. Nevertheless, recent population-genetic work indicates that the Sind-Kasur laboratory colony remains genetically close to its field-derived ancestors with only moderate divergence [47, 48]. This suggests that, despite long-term laboratory maintenance, the strain continues to retain essential genetic and biological features relevant to vector-parasite interactions and remains a good tool to use in vector experiments.

There are different approaches to achieve virus infections in mosquitoes under experimental conditions. Intrathoracic injection of viruses offers a powerful and reliable method for experimental viral infections, achieving high, near 100%, and reproducible infection rates across individuals, as shown in *Chapter 5*. Homogenous viral exposure and minimal variability between replicates makes this method highly suitable for laboratory studies, however this method does not mimic natural viral transmission. Based on current evidence, ISVs are often thought to be transmitted vertically, from infected females to offspring, and several studies support this as a key mechanism for establishing ISVs within mosquito colonies [49-53]. Future work should evaluate candidate viruses for their capacity for vertical transmission and stable maintenance of infection in mosquito colonies.

Translating the laboratory to field applications; Taking it out into the world

To ensure consistent and high infection rates in experimental settings, ISVs are commonly administered via intrathoracic injection, which delivers a controlled and equal dose of viral particles to each mosquito. However, injection is not a practical delivery method for field applications. For implementation in natural mosquito

populations, oral delivery, such as through sugar-based feeding stations, represents a more operationally attractive approach. This strategy takes advantage of the fact that mosquitoes regularly ingest sugar meals (e.g. from nectar or other plant sources) before or between blood-feeding events. Integrating ISVs into sugar-bait stations could be a viable approach, allowing field mosquitoes to ingest viral agents during sugar meal acquisition. However, this strategy requires careful optimization, including assessment of viral stability in sugar solutions and determination of the effective viral dose required to establish infection via ingestion. Previous studies have demonstrated that mosquito feeding stations can achieve high uptake rates. As example, baiting trials in Mali showed that indoor, single-bait-stations (one station per house) placed inside homes resulted in 28 – 53% of female *An. gambiae* s.l. and 37 – 78% of males fed on sugar [54, 55]. By contrast, field validation in Zambia used outdoor wall-mounted feeding stations for *Anopheles funestus* and *An. gambiae* mosquitoes and found a daily sugar-bait feeding rate of only 3.9 – 8.9% of the sampled population [56]. This indicates that the effectiveness of this delivery route for ISVs depends on factors such as mosquito species, environmental conditions, and feeding behavior. Further work is needed to confirm the viability and efficiency of oral delivery for a candidate virus in both laboratory and field-relevant settings.

In parallel with technical feasibility, ISV-based field applications must also navigate complex regulatory and ethical landscapes. Releasing viral agents into the environment requires risk assessment and biosafety approval by local and international regulatory bodies. Transparent communication with communities and ethical oversight are crucial components for responsible deployment. The necessity to ensure local ownership and support was painfully illustrated by the recent project in Burkina Faso, where the Target Malaria initiative ended the release of genetically-modified male mosquitoes to suppress the wild mosquito population. Despite years of research and collaboration, Burkina Faso's Ministry of Higher Education, Research and Innovation sealed all GMO (genetically modified organisms) containing enclosures and destroyed the gathered samples, following discontent and concerns about the GMO-based approach. This underscores the critical role of stakeholder consent, transparency and ownership [57]. For any biological intervention to be successfully implemented in natural settings, public acceptance is essential. This acceptance relies on demonstrable benefits, absence of adverse ecological or human health effects, and cost-effectiveness. Beyond this, long-term success also depends on the local community's understanding of the underlying science, the purpose of the intervention, and the perceived personal and public health benefits. Any novel intervention, such as a virus-based symbiont to reduce *Plasmodium* transmission, must be developed in tandem with strategies for community engagement, education, and transparency.

One potential advantage of viral symbiont-based approaches is that, once established in a population, they may require less direct citizen participation compared to methods such as bed net distribution, indoor residual spraying, or efforts to mobilize community members to remove or target mosquito breeding sites. This could reduce the logistical and financial burden of long-term maintenance, especially in low-resource settings. However, this does not imply that the method is entirely self-sustaining. Seasonal fluctuations in mosquito populations may require periodic reintroductions of the viral agent, and establishing some local support and regulatory approval will be essential. Regular entomological surveillance would be required to track viral and plasmodial infection prevalence, assess long-term trends in efficacy, and detect possible unintended ecological effects. If successful, such an approach could complement existing malaria control tools by reducing parasite development in the vector. Rather than replacing existing tools like insecticide-treated nets, ISV-based approaches would ideally function as part of an integrated vector management strategy.

Concluding remarks; The road from innovative concept to implementation

This thesis focusses on the use of an ISV as malaria interfering agent and discusses the possibility of this intervention strategy in *Chapter 1*, which shows that for an effective strategy many biological questions need to be answered. First and foremost, a suitable virus from the expanding mosquito virosphere must be found and deemed feasible of usage. An overview of the current mosquito virome is given in *Chapter 2*. We make a modest yet meaningful contribution to expanding the *Anopheles* virome knowledge in *Chapter 3* by generating our own small RNA sequencing data set and continue with experimental approaches in *Chapter 4 and 5*. We set up a *Plasmodium*-virus co-infection method and test multiple available ISVs for interference with malaria oocyst formation. If the best strategy would be to first explore the virosphere more or to experimentally challenge readily available ISVs we cannot say. Most importantly, such a novel malaria control strategy should be effective, safe and ideally self-sustaining. While there is potential for the use of ISVs as *Plasmodium* interfering agents, research is still in an early phase and much more research needs to be done before this method may be implemented. But whatever approach is chosen, each study conducted will bring us closer to a possible successful malaria elimination strategy. In an ideal scenario, a stably inherited ISV with no ecological side effects could be introduced seasonally via sugar bait stations in endemic regions, drastically reducing human infection rates without reliance on chemical controls.

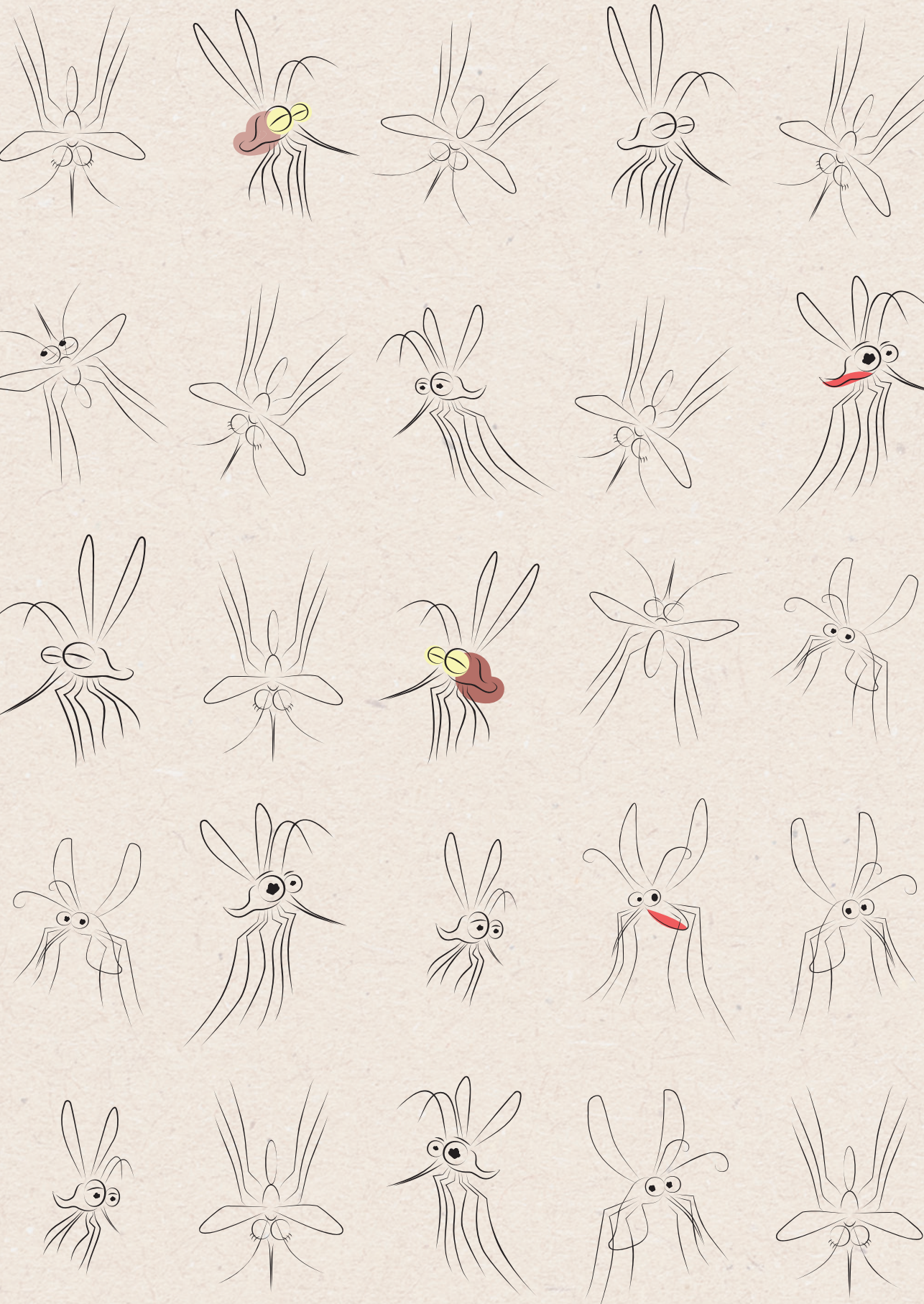
In my view, the ideal viral candidate would be insect-specific (preferably *Anopheles*-specific), would not affect mosquito viability or behavior, and would fully block *Plasmodium* transmission to mammalian hosts. While such a candidate has yet to be identified, our findings provide foundational insights and experimental tools to move towards this goal. The ISV-based strategy explored in this thesis represents a promising addition to the vector control toolbox, with the potential to complement or even replace chemical-based approaches that are increasingly undermined by resistance. In conclusion, ISV-based vector control represents a promising but complex strategy that requires multidisciplinary collaboration from molecular virology and entomology to social sciences and public health policy. As this field progresses, aligning scientific innovation with community trust, equitable access, and long-term ecological monitoring will be essential to ensure both efficacy and acceptance of future interventions.

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Appendix

Summary

Samenvatting

Research data management

List of Publications

Acknowledgements

Curriculum Vitae

Portfolio

Summary

Malaria remains one of the most significant vector-borne diseases worldwide, caused by *Plasmodium* parasites transmitted through the bites of infected *Anopheles* mosquitoes. Despite significant progress in lowering the disease burden, 597,000 malaria related deaths still occurred worldwide in 2023. Beyond their role as malaria vectors, *Anopheles* mosquitoes also harbour a wide variety of viruses, collectively known as the mosquito virome. Many of these viruses are insect-specific viruses (ISVs) that only infect insects. Increasing evidence suggests that ISVs can influence mosquito biology and possibly affect how mosquitoes transmit other pathogens. Understanding the *Anopheles* virome and the interactions between ISVs, mosquitoes and parasites may therefore open new possibilities for malaria control strategies by exploiting naturally occurring viral symbionts to reduce vector competence.

In *chapter 1*, we explored the possibility that ISVs might influence the transmission of *Plasmodium* parasites in *Anopheles* mosquitoes by reviewing the literature. Although direct evidence is lacking, viral infections can trigger immune pathways that are also known to act against parasites, suggesting that potential indirect virus-parasite interactions are possible. These tripartite interactions between virus, parasite, and mosquito could be used as a new strategy to reduce malaria transmission to lower the disease burden.

In *chapter 2*, we compiled a comprehensive database of ISVs from 175 studies over the last 25 years across the *Anopheles*, *Aedes* and *Culex* mosquito genera. We found that *Anopheles* hosts a diverse range of ISVs, but also that the *Anopheles* virome remains the least characterized among vector mosquitoes. The chapter also discusses and highlights the challenges of integrating data from different metagenomic studies and emphasizes the importance of standardized reporting to improve, compare, and utilize available mosquito virome data in future research.

Chapter 3 reports a small RNA-based virome analysis of *Anopheles gambiae* mosquitoes collected in the Goden regio of Burkina Faso. Sequencing revealed a diverse range of viruses, with the most common being a newly identified multipartite virus, which we named Goden partiti-like virus, detected in 10 of the 38 sampled mosquito pools. Additionally, we found *Aedes aegypti* To flavivirus-like sequences, likely representing an endogenous viral element (EVE), a fragment of a viral genome sequence that has become integrated into the host's genome, rather than an active infection. Overall, these findings expand our knowledge of the *Anopheles* virome and provide new leads for identifying ISVs that can be utilized for *Plasmodium* interference.

In *chapter 4*, we compared different ways of blood feeding *Anopheles* mosquitoes to identify the most reliable and practical system for experiments with *Plasmodium* transmission. Because this thesis also required a system that could support both *Plasmodium* and ISV infections within one mosquito, we aimed to identify a feeding setup that was not only efficient for parasite transmission but also suitable for viral co-infection experiments. We used either water-heated or electronically heated feeders, covered with parafilm or a collagen membrane to mimic human skin. Feeding rates, blood meal volumes and *Plasmodium* infection rates were measured. Although feeding rates and blood volumes were similar across both heating sources, mosquitoes fed through collagen membranes did not become infected with *Plasmodium*. Because of this, collagen membranes were excluded from all subsequent *Plasmodium* transmission assays and all further studies used electronically heated feeders covered with a parafilm membrane.

Chapter 5 experimentally investigated whether ISVs can influence *Plasmodium* parasite development and transmission in *Anopheles* mosquitoes. A panel of known ISVs was selected based on their availability and diversity, representing different viral families and genome types, to evaluate their ability to infect *Anopheles* hosts. We first assessed viral infectivity in *Anopheles* cell cultures, and those viruses that replicated successfully were subsequently tested in mosquitoes to evaluate their replication dynamics *in vivo*. Viruses that efficiently replicated without affecting mosquito mortality were used in *in vivo* ISV-*Plasmodium* co-infections to test if they affect parasite transmission. Unfortunately, no ISV was found to interfere with *Plasmodium* infection, but this work established a valuable framework for studying virus-parasite interactions in mosquitoes and identified several viruses that can be used as tools to further investigate *Anopheles* immunity.

While the earlier chapters focused on the *Anopheles* mosquito, we broadened our scope in *chapter 6* to *Aedes aegypti* (the yellow fever mosquito), a mosquito species that transmits human pathogenic viruses. The overall responses of mosquitoes to viral infection remain poorly characterized, and understanding how gene expression changes during infection is crucial for identifying conserved antiviral defense pathways across species. *Chapter 6* describes how in *Aedes aegypti* cells, a cluster of eight small heat shock proteins (sHsps) is induced in response to viral infection. This cascade acts early during viral infection and exhibits pan-antiviral activity, reducing chikungunya, Sindbis and dengue virus replication, but also has an antiviral effect against O'nyong-nyong virus in *Anopheles* cells. We propose that these sHsps are promising targets for novel antiviral strategies against arthropod-borne viruses transmitted by mosquitoes.

Finally, in the general discussion in *chapter 7*, I highlight the findings of the previous chapters, and emphasize the importance of understanding how ISVs might affect mosquito fitness, behaviour and ecology as well as their transmission dynamics and possible effects in different host species. I also discuss the potential of ISV-based approaches as vector control measures. Ultimately, ISV-based strategies could complement existing vector control strategies, though further research, validation and community acceptance are essential for safe and effective implementation.

Samenvatting

Malaria is nog altijd één van de belangrijkste door muggen overgedragen ziekten ter wereld. De ziekte wordt veroorzaakt door *Plasmodium* parasieten, die via de beet van een besmette *Anopheles* mug op mensen worden overgebracht. Ondanks grote vooruitgang in de bestrijding van malaria, stierven er in 2023 nog steeds ongeveer 597,000 mensen aan de gevolgen van deze ziekte. Naast hun rol als overbrenger van malariaparasieten, dragen deze *Anopheles* muggen ook een groot aantal virussen met zich mee, samen het muggenviroom genoemd. Veel van deze virussen zijn Insect Specifieke Virussen (ISVs), die alleen insecten besmetten en niet overgedragen worden op mensen of andere diersoorten. Steeds meer onderzoek laat zien dat deze ISVs invloed kunnen hebben op de biologie van muggen en mogelijk ook op hun vermogen om ziektes over te dragen. Door beter te begrijpen hoe deze virussen, muggen en parasieten op elkaar inwerken, kunnen we misschien nieuwe manieren vinden om malaria te bestrijden.

In *hoofdstuk 1* bekeken we of ISVs invloed kunnen hebben op de overdracht van malaria op basis van bestaande literatuur. Er is hiervoor nog geen direct bewijs, maar eerdere studies laten wel zien dat virale infecties het afweersysteem van muggen kunnen activeren. Deze activatie kan vervolgens misschien ook tegen parasieten werken. Dit suggereert dat virussen indirect misschien een rol kunnen spelen in het verminderen van malariaverspreiding.

In *hoofdstuk 2* verzamelden we gegevens uit 175 wetenschappelijke studies van de afgelopen 25 jaar over ISVs in verschillende muggensoorten: *Anopheles*-, *Aedes*-, en *Culex*-muggen. We ontdekten dat *Anopheles* muggen een grote diversiteit aan ISVs herbergen, maar ook dat het *Anopheles* viroom, vergeleken met de andere muggensoorten, nog het minst goed is onderzocht. Ook bespreken we de uitdagingen van het samenvoegen van gegevens uit verschillende studies. We benadrukken het belang van het op een eenduidige manier rapporteren van resultaten, zodat gegevens beter kunnen worden vergeleken en gebruikt in vervolgonderzoek.

Hoofdstuk 3 beschrijft een door ons uitgevoerde analyse van het viroom van *Anopheles* muggen, verzameld in de Goden regio in Burkina Faso. We vonden een grote verscheidenheid aan virussen, waaronder een nieuw ontdekt virus dat we de naam Goden partiti-like virus gaven. Dit virus werd gevonden in 10 van de 38 onderzochte muggenpools. Ook vonden we sporen van het *Aedes aegypti* To-flavivirus. Dit is waarschijnlijk geen actieve virale infectie, maar resten van oud viraal DNA die in het DNA van de mug zijn ingebouwd, een zogenaamd endogeen

viraal element. Deze resultaten breiden onze kennis van het *Anopheles* viroom uit en geven nieuwe aanknopingspunten voor het vinden van ISV's die de ontwikkeling van malariaparasieten kunnen verstoren.

In *hoofdstuk 4* vergeleken we verschillende manieren om *Anopheles* muggen met bloed te voeren om de meest betrouwbare en praktische methode te vinden voor experimenten met malaria-overdracht. Omdat we in dit proefschrift ook een systeem wilden ontwikkelen dat zowel malaria- als ISV-infecties gelijktijdig kan ondersteunen, onderzochten we ook welke voedingsopstelling het meest geschikt was voor deze co-infectie experimenten. We gebruikten met water of elektronisch verwarmde muggen voedingsapparaten, bedekt met parafilm of een collageenmembraan dat de menselijke huid nabootst. De aantallen muggen die voedden, het volume opgenomen bloed en de hoeveelheid malariaparasieten werden vervolgens gemeten. Hoewel de muggen evenveel bloed opnamen bij de verschillende methoden, was er geen overdracht van parasieten wanneer er een collageenmembraan gebruikt werd. Daarom besloten we om collageenmembranen niet meer te gebruiken voor vervolgstudies, maar parafilm toe te passen wanneer we malaria infecties uitvoeren.

In *hoofdstuk 5* onderzochten we of ISV's de ontwikkeling en overdracht van malaria in *Anopheles* muggen kunnen beïnvloeden. We selecteerden verschillende beschikbare ISV's uit diverse virusfamilies om te testen of ze replicateerden in muggen-cellijnen en levende muggen. De virussen die efficiënt vermenigvuldigden zonder dat de muggen eraan stierven, werden vervolgens samen met malaria infecties getest om te zien of ze invloed hadden op de malariaoverdracht. Geen van de virussen bleek effect te hebben op de ontwikkeling van de parasiet, maar het onderzoek legt wel een stevige basis voor toekomstig werk naar de interactie tussen virussen, parasieten en muggen.

In *hoofdstuk 6* breidden we ons onderzoek uit naar een andere belangrijke muggensoort, *Aedes aegypti*, de gelekoortsmug. Deze soort is een belangrijke overdrager van voor mensen gevaarlijke virussen zoals dengue, Zika en chikungunya. Hoewel er al veel onderzoek is gedaan naar antivirale afweer bij insecten, is er nog weinig bekend over hoe muggen op transcriptioneel niveau reageren op virale infecties. In dit hoofdstuk laten we zien dat er in *Aedes aegypti* cellen een groep van acht kleine heatshock-eiwitten (sHsps) geactiveerd werd als vroege afweerreactie op virale infectie. Deze eiwitten remden de vermeerdering van verschillende virussen, waaronder chikungunya-, Sindbis- en denguevirus in *Aedes* cellen, en bleken zelfs antivirale effecten te hebben tegen O'nyong-nyong virus in *Anopheles* cellen. Deze sHsps kunnen in de toekomst mogelijk dienen als nieuwe aangrijpingspunten voor antivirale strategieën tegen ziekten die door muggen worden overgedragen.

Tot slot brengt *hoofdstuk 7* de belangrijkste bevindingen uit alle eerdere hoofdstukken samen. Ik benadruk het belang van een beter begrip van hoe ISV's de gezondheid, het gedrag en de verspreiding van door muggen overgedragen ziekten beïnvloeden. ISV's zouden uiteindelijk kunnen worden ingezet als natuurlijke hulpmiddelen in de strijd tegen malaria, maar meer onderzoek, zorgvuldige beoordelingen en maatschappelijke acceptatie zijn noodzakelijk voordat zulke strategieën veilig kunnen worden toegepast.

Research data management

Data collection and storage

The research presented in this thesis was carried out at and stored in accordance with the rules and regulations of the Radboud University Medical Center for FAIR (Findable, Accessible, Interoperable, Reusable) research data management. All data, unless specified otherwise, were obtained through laboratory experiments and analyzed on the department servers. All data and analyses obtained during my PhD studies are stored digitally on the departmental server, which is backed up daily. Raw unaltered data were stored alongside processed files. The raw data incorporated in this thesis have been made accessible in an organized manner. Data and analyses files are saved per thesis chapter, and per figure on the departmental server. Experimental procedures and results have been documented in a physical lab journal and on Labguru, an electronic lab journal accessible to all members of the research group. *Anopheles* mosquito materials were shared in adherence to the Nagoya Protocol that provides a transparent legal framework for the fair and equitable sharing of benefits arising from resources. Documentation for material export was provided by representatives of the Burkina Faso Ministry of Health.

Data sharing

Chapters 1, 2, 4, 5 and 6 have been published Open Access and corresponding data files, ordered per chapter/publication, are stored in the departmental server and on the Radboud Data Repository with open access (Radboud Collection identifiers: tibvpam_to000760a_dsc_921, tcgdvm_to000761a_dsc_643, hemotek_to000763a_dsc_922, coinfect_to000764a_dsc_027 and shsps_to000765a_dsc_017). All raw sequencing data used in chapter 3 of this thesis is stored in the departmental server and has been deposited in the NCBI Sequence Read Archive (SRA) with the dataset identifier PRJNA1390778, to be released at 01-01-2027 or upon manuscript publication. Raw sequencing files, together with analyzed result files are also stored on the Radboud Data Repository (Radboud Collection identifier: srnaseq_to000762a_dsc_394) with restricted access, the data is available upon request from the principal investigator or upon manuscript publication, which we strive to do as soon as possible.

List of Publications

- **Schinkel M.**, Overheul G.J., Ploeger E., van Gemert G.J., Junglen S., Miesen P., Bousema T., van Rij R.P. Systemic infection of insect specific viruses does not affect sporozoite formation in *Anopheles* mosquitoes. (2025) PLoS Negl Trop Dis 19(12): e0013848. doi: 10.1371/journal.pntd.0013848.
- Blanken S.L., Inklaar M.R., Z. Wan Z., Evers F., Smit M., Kalyuzhnyy V., Verhoef J.M.J., Bekkering E.T., **Schinkel M.**, Mulder S., Andrade C.M., van Gemert G.J., Gusinac A., Zeeuwen P., Ederveen T.H.A., Bousema T., Hol F.J.H., Blood, sweat, and beers: investigating mosquito biting preferences amidst noise and intoxication in a cross-sectional cohort study at a large music festival bioRxiv 2025.08.21.671470; doi.org/10.1101/2025.08.21.671470.
- Qu J.^{*}, **Schinkel M.**^{*}, Chiggiato L., Rosendo Machado S., Overheul G.J., van Rij R.P. The Hsf1-sHsp cascade has pan-antiviral activity in mosquito cells. *Commun Biol* **8**, 123 (2025). doi.org/10.1038/s42003-024-07435-4.
- **Schinkel M.**, Bousema T., van Rij R.P. Tripartite interactions between viruses, parasites, and mosquitoes. *Curr Opin Insect Sci.* 2024 Aug;64:101222. doi:10.1016/j.cois.2024.101222.
- Moonen J.P.^{*}, **Schinkel M.**^{*}, van der Most T.^{*}, Miesen P., van Rij R.P. Composition and global distribution of the mosquito virome - A comprehensive database of insect-specific viruses. *One Health.* 2023 Jan 20;16:100490. doi:10.1016/j.onehlt.2023.100490.
- Graumans W.^{*}, **Schinkel M.**^{*}, van Gemert G.J., Spitzen J., Bousema T., Miesen P. Comparative analysis of glass and Hemotek membrane feeding systems for malaria transmission research. *Trans R Soc Trop Med Hyg.* 2023 Jun 2;117(6):476-478. doi:10.1093/trstmh/trac135.
- Vosseberg, J., **Schinkel, M.**, Gremmen, S., Snel, B. The spread of the first introns in proto-eukaryotic paralogs. *Commun Biol* **5**, 476 (2022). doi.org/10.1038/s42003-022-03426-5.
- Jiang L., **Schinkel M.**, van Essen M., Schiffelers R.M. Bacterial membrane vesicles as promising vaccine candidates. *Eur J Pharm Biopharm.* 2019 Dec;145:1-6. doi:10.1016/j.ejpb.2019.09.021. Epub 2019 Sep 24.

* Shared first authorship

Acknowledgements

Although there is only one name on the cover, this journey would have been impossible without the help of many colleagues, friends and family. This section is dedicated to all of you who helped bringing this work to life, in one way or another.

I would like to express my sincere gratitude to my promotors for their guidance, support, and trust throughout my PhD journey. Your approaches were sometimes different in style and perspective, yet precisely this balance challenged me, strengthened my work, and helped me grow as an independent researcher. I am deeply thankful for your time, encouragement and sometimes much needed patience. I would also like to thank the members of my manuscript committee for taking the time to carefully read this work and for their valuable feedback. I am grateful to all the colleagues and students who contributed to the chapters of this thesis, whether through experiments, discussions, data analysis, or constructive feedback; your input significantly enriched this work. Finally, I would like to sincerely thank our collaborators in Africa for their essential support in mosquito collection and fieldwork. Their local expertise, dedication, and collaboration were indispensable to this research.

I would like to thank my paranims, my closest colleagues, and dearest friends, with whom I shared the best office imaginable, filled with chocolate, KFC lunches, sushi dates, laughter, and endless support. Not only during office hours, but far beyond them. From horse riding trips and spontaneous shopping sprees to cherry blossom viewing and zoo adventures, our time together has been full of unforgettable moments that made this journey not only easier but genuinely joyful and I am happy to have you two by my side.

I would like to thank all members, past and present, of the Virology and Parasitology group. It is impossible to name everyone individually, but I am deeply grateful to each of you for the stimulating scientific discussions, practical support, and the open atmosphere in the lab. Beyond the research itself, the many shared activities and traditions made this PhD a truly memorable experience. From exchanging plant cuttings, BSL3 foute party injection experiments, writing retreats with lama walking, lab-Sinterklaas celebrations, wheelbarrow rides, Lowlands Science, salsa dancing, back garden camping parties, office dog-sitting, massive seafood platters in France, pimp coats, a costume dress up party in Istanbul and many more.

I would also like to express my heartfelt thanks to the many friends I have met along the way throughout my studies, internships, and PhD journey. Together, we

grew from carefree, beer-drinking and dancing bachelor students, to Cards Against Humanity playing master interns, and eventually into people with “grown-up” jobs and the first signs of aging. Thank you for the wonderful dinners, the memorable horse-riding adventures at the stables every weekend or online on sso, and, above all, for your constant moral support, especially during the many moments I found myself complaining about work.

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Ik wil mijn kat Uk bedanken, mijn meest trouwe luisteraar, die elke dag geduldig mijn samenvattingen aanhoorde zodra ik thuis kwam van werk. Ze bood eindeloze morele steun door op mijn bureau te gaan zitten en precies aandacht te eisen wanneer de deadlines naderden of wanneer ik een online meeting had.

Mijn grootste dank gaat uit naar mijn partner Thijs. Als mede-PhD-student begrijpt hij als geen ander de uitdagingen van het wetenschappelijke leven: de belachelijke uren die we soms maken, in het weekend moeten werken, of midden in de nacht een presentatie voorbereiden zodat de laatste resultaten er nog in kunnen. Samen wetenschapper zijn betekent dat we niet alleen elkaar begrijpen, maar elkaar ook inspireren en mogelijk een Schinkel-Conner lab kunnen beginnen. Van avontuurlijke reizen ver weg tot spannende avonturen thuis in Baldur's Gate, van het dragen van een kimono in Japan tot in onze trainingsbroek op de bank. Bedankt dat je niet alleen mijn partner bent, maar ook mijn favoriete collega in de wetenschap en de chaos van het leven.

Curriculum vitae



Michelle Schinkel was born on February 16, 1994, in Vlissingen, Zeeland the Netherlands. She grew up in Zeeland and graduated from Nehalennia (VWO High School) in 2013. After this, she began her undergraduate studies in Biomedical sciences in 2013 at Utrecht University, the Netherlands. During her bachelor's thesis in the department of NMR spectroscopy at Utrecht University, she studied the epidermal growth factor receptor and its interaction with the extracellular matrix through solid-state NMR under the supervision of Prof. Dr. Marc Baldus. She obtained her bachelor's degree in 2016.

After obtaining her bachelor's degree, Michelle worked for nine months at the Department of Oncological Haematology of the Utrecht Medical Centre (UMC) in Utrecht as a medical secretary. In September 2017, she started her master's degree in Molecular and Cellular Life Sciences at Utrecht University. Her first Master internship was in the Department of Medical Microbiology of Utrecht University under the supervision of Prof. Dr. Hans de Cock. During this internship, Michelle studied *Aspergillus fumigatus* conidia and their protection against oxidative stress. For her work during this internship, she was awarded with the Unilever Research Prize, a prestigious annual award given to thirteen talented master's students from Dutch Universities for their outstanding research.

Her second master's internship took place in the Department of Theoretical biology and Bioinformatics at Utrecht University. Here, she worked under the supervision of Prof. Dr. Berend Snel in his Evolutionary Genomics and Integrative Bioinformatics group on a project about the spread of the first introns in proto-eukaryotic paralogs. Her internship in the bioinformatics department together with in-depth bioinformatic courses awarded her with a minor in bioinformatics. She completed her master's degree in September 2019.

In October 2019, Michelle moved to Nijmegen to begin her PhD studies in the group of Prof. Dr. Ir. Ronald van Rij. Her doctoral research focused on the virome of *Anopheles* mosquitoes and its possible effects on *Plasmodium* transmission, which is captured in this thesis. Following her PhD, she continued for 2 years as a postdoctoral researcher in the same research group, working on pandemic preparedness, focussing on induced Pluripotent Stem Cell-derived culture models to assess virulence of viruses

with pandemic potential. Currently, Michelle is working as a specialist International vaccine Seed Management within the BioTechnology Solutions team at MSD, animal health, Boxmeer.



Portfolio

Department: Medical Microbiology
PhD period: 15/10/2019 – 14/12/2024
PhD Supervisor(s): Prof. dr. ir. R.P. van Rij, Prof. dr. T. Bousema
PhD Co-supervisor(s): Dr. P. Miesen

Training activities	Hours
Courses	
• Endnote workshop (2019)	2.80
• Literature review for your PhD (2019)	5.60
• Adobe illustrator (2020)	2.80
• RIMLS - Introduction course "In the lead of my PhD" (2020)	15.00
• RIMLS PhD course (2020)	21.00
• Meet the expert: RNA-seq experiment design (2020)	2.80
• Radboudumc - Scientific integrity (2020)	20.00
• Statistiek voor promovendi met SPSS (opfriscursus) (2021)	56.00
• Open Science for PhD candidates (2021)	28.00
• Course in virology Erasmus MC (2021)	40.00
• RU - Mindfulness Based Stress Reduction (2022)	45.00
• RU - Een PhD en dan? Solliciteren, netwerken en je loopbaan onder de loep (2023)	20.00
• The next step in my career (2023)	16.00
• RU - The Art of Finishing Up (2023)	10.00
• ICTV/EVBC Workshop on Automating Virus Taxonomy (2023)	8.00
Seminars	
• Veupathdb seminar1 (2020)	2.80
• Veupathdb seminar2 (2020)	2.80
• Veupathdb seminar3 (2020)	2.80
• Veupathdb seminar4 (2020)	2.80
• Veupathdb seminar5 (2020)	2.80
• Research integrity round (2021)	1.50
• ERC viromics lecture (2023)	1.00
• ERC viromics lecture (2023)	1.00
• Research integrity round (2023)	1.50
• Meet the expert - How to prepare for your PhD defense (2024)	1.00
Conferences	
• Annual science day centre of infectious diseases (2019)	7.00
• American Society for Virology (AVS) (digital) (2020)	7.00
• Arthropod Genomics Symposium (digital) (2020)	21.00
• RIMLS PhD retreat (2021) *	8.00
• Radboudumc New frontiers symposium (2021) *	24.00
• Dutch Annual Virology Symposium (DAVS) (2022)	8.00
• Radboud Centre for Infectious diseases (RCI) science day (2022)	8.00
• RIMLS PhD retreat (2022) *	24.00
• Dutch Arbovirus Research Network meeting (DARN) (2022)	8.00
• Dutch Annual Virology Symposium (DAVS) (2023) *	16.00
• RIMLS PhD retreat (2023) #	24.00
• Dutch Arbovirus Research Network meeting (DARN) (2023)	8.00
• Roscoff insect models for infection biology (2023) #	48.00
• ICTV/EVBC Automating Virus Taxonomy (digital) (2023)	16.00
• Netherlands malaria day (2023)	8.00
• PDPC symposium (2023)	8.00

Training activities	Hours
Other	
• Journal clubs medical microbiology 2019 (2019)	27.00
• Journal clubs medical microbiology 2020 (2020)	105.00
• Journal clubs medical microbiology 2021 (2021)	69.00
• Journal clubs medical microbiology 2022 (2022)	66.00
• Lowlands science (2023)	48.00
• Journal clubs medical microbiology 2023 (2023)	54.00
• Journal clubs medical microbiology 2024 (2024)	9.00
Teaching activities	
Supervision	
• Supervision researchprojecten 2e jaars GNK/BMW (2021)	1.75
• Supervision master internship (2021)	56.00
• Supervision master internship (2022)	56.00
• Supervision master internship (2023)	56.00
Total	1130.75

Poster and oral presentations are indicated with * and #, respectively.





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