UNIVERSITY OF CALIFORNIA
RIVERSIDE

# The piRNA Pathway in the Mosquito *Aedes aegypti*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular and Developmental Biology

by

Sally Waithera Ireri

December 2023

Dissertation Committee:
Dr. Peter Atkinson, Chairperson
Dr. Ted Karginov
Dr. Juliet Morrison
Dr. Naoki Yamanaka

The Dissertation of Sally Waithera Ireri is approved:

_____

_____

_____

_____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor Dr. Peter Atkinson for allowing me the space to discover a love for genetics in his lab, while studying mosquitoes. I would also like to acknowledge my department, Cell, Molecular and Developmental Biology (CMDB) for awarding me a Dissertation Year Fellowship, and the other institutions that funded the work presented in this dissertation, the National Institutes of Health, the Centers for Disease Control and Prevention, and the Pacific Southwest Center for Excellence in Vector-Borne Diseases.

I would like to thank the scientists who set me on this path, including Mrs. Muse from high school Chemistry in Kianda, Dr. Christine Adhiambo and Dr. Edward Muge from the University of Nairobi Department of Biochemistry, and Dr. Daniel Masiga from the International Center for Insect Physiology and Ecology (ICIPE). Thank you to my committee members at UCR: Dr Ted Karginov, Dr Naoki Yamanaka and especially Dr Juliet Morrison; and Dr. Linda Walling, who despite not being on my committee always made time for me. I am grateful for your time and the technical help you provided during my PhD.

I want to thank my village, both in the US and back home in Kenya, including my first lab mates in the US, Dr. Anna-Louise Lee Doss, Dr Baoju Wang, and Simran Sandhu. Thank you to my cohort-mates Jordan Lillibridge and Dr Frantzeska Giginis. It's been a pleasure going through the journey of graduate school with you. Thank you to my therapist, Vivian, you were invaluable.

A big thank you to my friends Eva Otwoma, Kawira Mathenge, Mwende Mwololo, Wanjiru Mutero, Kathleen Siminyu, Sarah Wanjohi, Aggie Ogallo for always being there for me. Thank you to my partner Yusuph Mavura, my cheerleader. And finally, thank you to my parents, David and Catherine Ireri, and my brothers Philip and Njuguna Ireri. I would not be here without your continuous support.

ABSTRACT OF THE DISSERTATION

The piRNA Pathway in the Mosquito Aedes aegypti

by

Sally Waithera Ireri

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, December 2023
Dr. Peter W Atkinson, Chairperson

The development of efficient techniques to interrupt the transmission of human pathogenic arboviruses such as Dengue, Zika and West Nile virus is of vital importance as current mosquito control strategies are not sufficient. Insect protection from viruses is typically carried out by the siRNA pathway which involves the degradation of foreign genetic material during an active infection.

Recently, another class of small RNAs that form the piRNA pathway have been implicated in antiviral activity in *Aedes aegypti*. However, *Ae. aegypti* was discovered to express piRNAs somatically with recent confirmation of the antiviral activity of piRNAs in the ovaries lending credence to this theory. Not much is known about how the associated effectors of this pathway, known as the PIWI class of proteins, participate in this antiviral activity as most studies to date have been done in cell culture. To this end, I used CRISPR-Cas9 to knockout Ago3, Piwi4, Piwi5, Piwi6, and Piwi2 and characterized the resulting phenotypes. Knockout of each piwi gene led to a general reduction in viability and fertility of the G0 mosquitoes compared to the controls.

Immunoblotting for each tested Piwi protein in the larval midgut, compared to adult testes and blood-fed ovaries, revealed that Ago3 is not expressed in the larval stage or the testes, indicating the absence of the ping-pong amplification pathway in these tissues. This suggests that the piwi genes have developed functional specialization though further work *in vivo* is necessary to tease apart the potential functions.

The prevalence of endogenous viral elements (EVEs) from which piRNAs arise, in the *Ae. aegypti* genome, prompted the attempt to generate transgenic mosquitoes primed with immunity to an arbovirus of medical importance. This was done by locating and targeting unique loci in a highly repetitive piRNA cluster that is highly expressed in the midgut, to knock in a segment of the Sindbis virus, and test for SINV-piRNA expression. Establishment of the transgenic line using CRISPaint proved to be challenging, though successful integration was observed. Further investigation of this pathway could result in valuable information on new targets for genetic control of this vector species.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# CHAPTER ONE

## INTRODUCTION & BACKGROUND

### PART I: The Mosquito *Aedes aegypti*

**The mosquito *Aedes aegypti* and the diseases transmitted.**

The mosquito *Aedes aegypti*, also known as the yellow fever mosquito, is an insect in the order Diptera and belongs to the family Culicidae. Culicidae mosquitoes are further divided into Anopheline and Culicine sub-families, and *Aedes aegypti* belongs to the latter.

The *Ae. aegypti* mosquito is thought to have originated in sub-Saharan Africa where it still exists in its "sylvan" form referred to as *Ae. aegypti formosus* (Aaf) (Derryberry and Gartrell 1952, Tabachnick 1991, Crawford, Alves et al. 2017). This mosquito lives in forested areas and is primarily zoophilic (Lounibos 1981). In the Sahel region of Western Africa, the climate changed at the end of the Africa Humid Period resulting in a somewhat abrupt drying of the Sahara (Shanahan, McKay et al. 2015). The domesticated form of this mosquito, which is anthropophilic (Crawford, Alves et al. 2017) and has adapted to breed in containers in urban environments arose during this period, around 5000 years ago (Rose, Badolo et al. 2023). This domesticated mosquito is formally referred to as *Aedes aegypti aegypti* (Aaa) (Tabachnick 1991), but unless otherwise stated, *Ae. aegypti* will refer to the domesticated form Aaa. The domesticated *Ae. aegypti* was likely exported to the Americas

along with enslaved people aboard the slave ships plying the Atlantic Ocean between the West African coast and the Caribbean (Tabachnick 1991, Rose, Badolo et al. 2023). From there the yellow fever mosquito likely spread West across the Pacific Ocean and into Asia and Australia (Powell and Tabachnick 2013).

The mosquito feeds during the day, prefers to feed on humans and traditionally rests and feeds indoors, all of which increases the probability of interactions with people. However, recent reports have shown that *Ae. aegypti* is adapting to feed both indoors and outdoors, increasing the pool of potential hosts for blood-feeding and therefore having implications for the transmission of mosquito-borne diseases. (Ngugi, Mutuku et al. 2017).

The life cycle of *Ae. aegypti* is as follows (Fig.1.1): gravid females lay eggs singly in water holding containers in urban and peri-urban areas (Ngugi, Mutuku et al. 2017, Ouédraogo, Toé et al. 2022). The eggs laid can undergo desiccation if allowed to develop for a couple of hours in a moist environment before drying out. Water and a reduction of oxygen caused by the presence of microbial activity, and hence vegetation, in the water serve as a hatching signal (Christophers 1960).

The egg cap breaks open letting the L1 larvae wriggle free of the rest of the egg casing. The larvae mature and begin feeding, passing through four larval stages with feeding highest in L3 and L4 larvae where they will grow rapidly. The larvae molt and shed their exoskeleton as they progress through each larval developmental stage (Christophers 1960).

Male mosquitoes eclose from the pupal casing before the females, and within 48 hours of eclosing, the males and females are ready to mate. Female *Ae. aegypti* mosquitoes are mostly monandrous, i.e., the female will be inseminated once then become refractory to further insemination, however studies have shown that a small proportion of already mated females may mate again. (Gwadz, Craig et al. 1971, Degner and Harrington 2016). Mating has been found to occur close to the host (W. K Hartberg 1971) or in large swarms that typically occur at dawn and dusk and comprise of thousands of males to which females are attracted for mating (Hartberg 1971, Cabrera and Jaffe 2007, Fawaz, Allan et al. 2014).

Mature adult females can take several blood meals from different hosts during one gonadotrophic cycle (Scott, Clark et al. 1993). After a blood meal the females rest while egg development occurs. One female can typically around 200 eggs in up to five gonadotrophic cycles throughout her lifetime (Christophers 1960).

*Ae. aegypti* is an efficient vector of arthropod-borne viruses (arboviruses) such as Dengue virus, yellow fever virus (YFV), Chikungunya virus (CHIKV), Zika virus (ZIKV) and West Nile virus (WNV).

The extreme weather events associated with climate change have been shown to affect local mosquito population abundance and by extension the incidence of vector borne diseases in the local population (Nosrat, Altamirano et al. 2021), a problem that will be compounded

by the predicted increase in the range of *Ae. aegypti* due to climbing temperatures and shorter winters (Messina, Brady et al. 2019, Ryan, Carlson et al. 2019).

*Ae. aegypti* have spread throughout the state of California, after first being detected in three central California counties in 2013, and initial eradication efforts were unsuccessful as the mosquitoes were detected in six more counties the following year (Metzger, Hardstone Yoshimizu et al. 2017). Genotyping and microsatellite analysis of the invasive mosquitoes indicated two likely introductions of *Ae. aegypti* into California, likely occurring before the mosquitoes were detected by state vector control officials (Pless, Gloria-Soria et al. 2017, Lee, Schmidt et al. 2019)

Current vector control strategies

One of the oldest forms of vector control is environmental management to remove potential habitats for mosquito breeding with reports from Ancient Greece of the correlation between episodic fevers and swampy areas (Wilson, Courtenay et al. 2020). Other early interventions which showed success in reducing the transmission of vector-borne diseases include screening doors, windows, and chimneys to prevent entry of mosquitoes into the home (Takken, Snellen et al. 1990, Ferroni, Jefferson et al. 2012).

Chemical insecticides have been in use for at least 100 years as a method of control for agricultural and medically important pests. Resistance to insecticides among some insect vectors soon became a problem after widespread coordinated use of insecticides in the earlier

half of the 20th century. Dichloro-diphenyl-trichloroethane (DDT), a popular insecticide, was used to eliminate anopheline mosquitoes, and therefore malaria, in several regions around the world such as the South-eastern US and Northern Egypt (Shousha 1948, Derryberry and Gartrell 1952, Nájera, González-Silva et al. 2011) before it was abandoned due to the discovery that DDT and its metabolites are toxic and still persist in soil, contamination challenge decades after this insecticide was abandoned (Sudharshan, Naidu et al. 2012). By the 1960s scientists were aware of resistance to most major mosquito chemical insecticides, especially pyrethrins and organophosphates, and that this resistance was heritable therefore posing a significant risk to the efficacy of the insecticides (World Health Organization 1964, Moyes, Vontas et al. 2017). As use of chemical insecticides spread globally, reports of resistance to the four main classes of chemical insecticides, carbamates, pyrethrins, organo-chlorine and organo-phosphate derived insecticides cropped up with resistance to all four classes detected globally in nineteen out of 88 countries that reported to the WHO over the period of ten years ending in 2020 (Elissa, Mouchet et al. 1993, Moyes, Vontas et al. 2017, Jangir and Prasad 2022).

Chemical insecticides are applied in various ways, mainly via indoor residual spraying (IRS) insecticide-treated bed-nets and treating aquatic habitats with larvicides. The most common class of insecticide used worldwide for the control of arthropod-borne diseases is pyrethroids, despite the widespread development of resistance to this class of chemicals (Moyes, Vontas

et al. 2017, van den Berg, da Silva Bezerra et al. 2021). Resistance to chemical insecticides is on the rise in different regions globally (Gan, Leong et al. 2021, Ayettey, Ablorde et al. 2023, Tungu, Kabula et al. 2023) pointing to the urgent need for new interventions in mosquito control.

The Sterile insect technique (SIT) is a type of population suppression vector control which has been successfully employed in the eradication of agricultural pests such as the screwworm fly (Bushland and Hopkins 1951, Lindquist, Abusowa et al. 1992). Initially, sterility was induced in male arthropods using chemicals (Breeland, Jeffery et al. 1974), though this practice was eventually abandoned due to environmental safety concerns (Bořkovec 1976). X-rays replaced chemicals and were used to irradiate adult male mosquitoes rendering them sterile, then releasing large amounts of the sterile males and allowing them to mate with females in the wild causing a population crash (Knipling 1979).

Irradiation of the male spermatozoa generates chromosome breaks which are quiescent until mitotic divisions begin in the developing embryo which frequently generate errors during the repair process that result in dominant lethal mutations (Smith and von Borstel 1972). These mutations typically have a deleterious effect on zygotic development but could also produce a lethal phenotype in the later stages of juvenile development (Atwood, Von Borstel et al. 1956, Robinson 2005). Variations of SIT in mosquitoes have been attempted in various regions of the world and to varying degrees of success, but this technology never achieved

large-scale population reduction, and is greatly hampered by the need for continuous releases of sterile males (Laven, Cousserans et al. 1972, Kittayapong, Ninphanomchai et al. 2019, Kittayapong 2021).

Various variations of SIT have been proposed and tested, including a conditional version of SIT named "Release of Insects carrying a Dominant Lethal gene (RIDL)" which has been employed in the control of *Ae aegypti*. A strain of mosquitoes with a repressible gene that is lethal in the pupal stage of the mosquito was created by *piggyBac*-mediated transformation and named OX513A. (Phuc, Andreasen et al. 2007, Harris, Nimmo et al. 2011). *Ae. aegypti* mosquitoes carrying the lethal dominant gene have been released in the field at small scales where they have shown efficient population suppression (Harris, McKemey et al. 2012, Carvalho, McKemey et al. 2015).

One of the largest releases occurred in Jacobina, Brazil where a fluorescently tagged version of the OX513A strain of mosquitoes was released over a period of 27 months (Evans, Kotsakiozi et al. 2019). Extensive genotyping of both the OX513A and the local strains of *Ae. aegypti* mosquitoes in Jacobina, allowed for genetic monitoring to take place, which uncovered that extensive introgression of the OX513A genome had occurred with 10% - 60% of mosquitoes sampled testing positive for the introduced strain (Evans, Kotsakiozi et al. 2019). Though no transgenes were found in the sampled mosquitoes, this degree of introgression is cause for concern, as the hybridizing mosquitoes may result in an overall

population increase, souring the public's view of genetically modified mosquitoes as a tool of vector control (Servick 2019, Leftwich, Spurgin et al. 2021)

The development of CRISPR-Cas9 and site-specific gene editing ignited interest in homing endonuclease gene drives, which is an attractive area of research, as it allows for super-Mendelian inheritance, "driving" the desired gene into the population in a few generations (Deredec, Burt et al. 2008, Hammond, Galizi et al. 2016, Li, Yang et al. 2021) . While cage trials of gene drive systems have been attempted with both *Ae. aegypti, An. stephensi* and *Anopheles gambiae*, (Gantz, Jasinskiene et al. 2015, Kyrou, Hammond et al. 2018, Li, Yang et al. 2020, Hammond, Pollegioni et al. 2021, Li, Yang et al. 2021), there has only been one attempt so far in releasing CRISPR-Cas9 genetically modified sterile male mosquitoes into the field, where *An. gambiae* mosquitoes were released in a village in Burkina Faso (Yao, Millogo et al. 2022).

Population suppression using CRISPR-Cas9 paired with a homing gene drive has proven to be difficult to implement as the site-specificity of CRISPR-Cas9 can be overcome by the random development of mutations and selection for the resistant alleles at the target site (Hammond, Kyrou et al. 2017, Unckless, Clark et al. 2017).

Alleles resistant to the gene drive can form initially either in the parental germline or in the embryo due to maternally deposited Cas9, when the double-stranded break caused by Cas9 is repaired by non-homologous end joining (NHEJ) or microhomology-mediated end joining

(MMEJ) resulting in the formation of indels at the sgRNA target site (Champer, Reeves et al. 2017, Li, Yang et al. 2020). Genetic factors that abolish recognition by the sgRNA, and therefore cleavage by the nuclease, can result in resistant alleles which are strongly selected for due to the fitness costs of the drive, including relatively rare events such as de novo mutations, and common challenges such as genetic variation in the field populations (Unckless, Clark et al. 2017, Price, Windbichler et al. 2020).

Research is ongoing to prevent the development of resistant alleles through methods such as the careful choice of a highly conserved target site, which may indicate that any disruptions to the homing gene drive target site would be lethal, a technique that has been implemented successfully in small cage trials in *An. Gambiae* and *D. suzukii* (Kyrou, Hammond et al. 2018, Yadav, Butler et al. 2023). Further large indoor cage trials in *An. Gambiae* using the same construct showed complete population suppression in approximately 9 months without the development of resistance, a promising prospect for the control of malaria, the deadliest mosquito-borne disease (Hammond, Pollegioni et al. 2021). Similarly conserved targets in *Ae. aegypti* have been identified and are under investigation (Navarro-Payá, Flis et al. 2020) for inclusion in toxin-antidote types of gene drives under development (Kandul, Liu et al. 2019, Kandul, Liu et al. 2021).

Wolbachia is a genus of matrilineally transmitted intracellular bacterial endosymbionts found in the reproductive tissues of arthropods and have been implicated in a phenomenon

known as cytoplasmic incompatibility (CI) which arises when infected males are mated with uninfected females, resulting in non-viable offspring (Rousset, Bouchon et al. 1997, Hedges, Brownlie et al. 2008). Wolbachia is estimated to infect approximately half of all terrestrial arthropods (Weinert, Araujo-Jnr et al. 2015), where it alters sex ratios in the host organism in its favor, typically by CI, resulting in increased proportions of infected females in the population (reviewed in(Werren, Baldo et al. 2008). Among mosquitoes, while Wolbachia is naturally found in *Culex pipiens* worldwide (Yen and Barr 1973), most populations of *Ae. aegypti* do not naturally carry these bacteria (Gloria-Soria, Chiodo et al. 2018, Balaji, Jayachandran et al. 2019), while the Asian Tiger mosquito, *Ae. albopictus*, typically carries two strains of Wolbachia (AlbA and AlbB) (Sinkins 1995).

The molecular basis for CI was relatively recently established in *D. melanogaster*, where two genes from a bacteriophage known as WO that are expressed in the Wolbachia genome affect the reproductive potential of the arthropod host in a complex tangle/an interesting three-way interplay. These prophage WO-derived genes, *cifA* and *cifB* have been shown to result in CI when expressed in the germline of uninfected fly males that are mated with uninfected females (LePage, Metcalf et al. 2017). The discovery of these genes, also known as *cidA* and *cidB* allows for the development of more tailored approaches to manipulating reproductive fitness in arboviral vectors which are currently being explored (Beckmann, Ronau et al. 2017, Deehan, Lin et al. 2021).

Expression of these genes in *An. gambiae* mosquitoes resulted in the CI phenotype where *cifA* and *cifB* both induce CI with wild-type uninfected females though *cifB* alone is sufficient to cause CI while *cifA* was sufficient to rescue the CI phenotype (Adams, Abernathy et al. 2021). Investigations in *Cx. pipiens* Wolbachia genomes, shows that the *cidA* and *cidB* operon are present in varying copy numbers, acting in a toxin-antidote manner (Bonneau, Atyame et al. 2018).

Wolbachia is being investigated as a potential population suppression tool with field trials having been carried out in both *Ae. aegypti* and *Ae. albopictus* (O'Connor, Plichart et al. 2012, Zheng, Zhang et al. 2019, Crawford, Clarke et al. 2020). While the release of Wolbachia-infected *Ae. aegypti* males causes a significant decrease in mosquitoes in the treated area, the population eventually rebounds mostly due to migration of new mosquitoes into the area (Crawford, Clarke et al. 2020).

Infection and superinfection with various Wolbachia strains in laboratory settings has shown a marked reduction in the transmission of arboviruses of medical importance such as Dengue and Zika (Hoffmann, Montgomery et al. 2011, Ferguson, Kien et al. 2015, Ant, Herd et al. 2018) making this an attractive tool currently under investigation to control the spread of these arboviruses (Schmidt, Barton et al. 2017, Zheng, Zhang et al. 2019), though much remains to be seen regarding the stability of different Wolbachia strains in one host genome (Ant and Sinkins 2018).

The mechanism through which blocking of viral transmission by the mosquito occurs is unknown, with some hypothesizing that Wolbachia may perturb lipid metabolism, particularly cholesterol homeostasis, therefore preventing viral replication (Geoghegan, Stainton et al. 2017). The first successful population replacement of *Ae. aegypti* with Wolbachia-infected mosquitoes occurred in Cairns, Australia where releases beginning in 2011 have resulted in a corresponding significant reduction in Dengue virus transmission over the 8-year period since the study began (Ryan, Turley et al. 2019).

## PART II: SMALL RNA silencing pathways

There are three main small non-coding RNA pathways in insects. All three pathways rely on the interaction between specific small RNAS and an Argonaute protein to effect silencing of the target single-stranded RNA.

### The micro-RNA pathway

The first microRNA (miRNA) was discovered in *Caenorhabditis elegans* as gene regulatory RNAs important for development (Bartel 2004, Kim, Han et al. 2009). Transcription from endogenous sources *via* RNA polymerase II results in primary micro-RNAs which are processed by the Drosha protein in the nucleus and exported to the cytoplasm for further processing by the Dicer-1 protein resulting in mature duplex miRNA. One strand of the miRNA is loaded onto the Argonaute-1 (Ago-1) protein forming the micro-RNA-

interference silencing complex (mi-RISC) (reviewed in (Carthew and Sontheimer 2009) and (Kim, Han et al. 2009).

The main function of miRNAs is gene expression regulation. This occurs by recognition and binding to mRNA in the cytoplasm followed by cleavage of the target mRNA by Ago-1. However, miRNAs have been implicated in the *Ae aegypti* response to viral infection as well as the viral response to the mosquito immune system. (Hussain, Torres et al. 2012, Asgari 2014, Miesen, Ivens et al. 2016).

The small interfering RNA pathway

The siRNA pathway comprises of Dicer-2 and Argonaute-2 (Ago-2) as the catalytic proteins in the RNA-interference silencing complex (RISC) (van Rij, Saleh et al. 2006). Recognition and processing of viral RNA occurs in the cytoplasm where the pathway is activated by double-stranded RNA (dsRNA), which is formed at some stage during infection – usually the replication of the viral genome. Dicer-2 protein processes the viral RNA into siRNA duplexes – 19 bp double stranded RNA with 2 nucleotide 3' overhangs. The guide strand of the siRNA duplex is bound by the Ago-2 protein while the complementary strand is digested by an exoribonuclease. The methyltransferase, DmHen1 is responsible for the 2-O-methylation of the 3' end of mature siRNAs. The mature siRNAs are bound to Ago-2 which forms the siRNA RISC. This RNA-protein complex is responsible for the recognition and cleavage of dsRNA in the cytoplasm. The guide strand base-pairs with the free dsRNA which

is cleaved between the 10th and 11th nucleotides by Ago-2 endo-nuclease activity. The double-stranded RNA is further degraded by cytoplasmic nucleases. (Reviewed in (Blair and Olson 2015) & (Bonning and Saleh 2021)).

The piRNA pathway

The piRNA pathway comprises of a sub-family of Argonaute proteins known as the PIWI group of proteins. The piRNA system in an organism usually comprises of at least one Argonaute-3 (Ago3) homolog, one or more Piwi protein homologs and the associated small RNAs known as piRNAs.

The piRNA pathway was first discovered an important factor for male sterility in *D. melanogaster*(Aravin, Naumova et al. 2001). The piRNAs were first referred to as repeat-associated silencing small RNAs (rasi-RNAs) and arose from the suppressor of the *stellate* locus which was implicated in maintaining germline stability (Aravin, Naumova et al. 2001). Further investigations revealed that rasi-RNAs are associated with the Piwi clade of Argonaute proteins and are a distinct species of small RNAs, leading to the renaming to piRNAs, i.e., Piwi-interacting RNAs (Aravin, Lagos-Quintana et al. 2003, Aravin, Gaidatzis et al. 2006, Vagin, Sigova et al. 2006).

piRNAs are produced from distinct genomic loci known as piRNA clusters. Most of the clusters are located in the repetitive regions of the genome containing large segments of repetitive sequences and transposable element fragments while some piRNA clusters are

found in genic regions too, such as the untranslated regions of genes (Nishida, Saito et al. 2007, Robine, Lau et al. 2009, Saito, Inagaki et al. 2009). These small RNAs are 23-31 nucleotides in length and are 2'-O-methylated at the 3' end, a characteristic feature of piRNAs (Saito, Sakaguchi et al. 2007).

A seemingly conserved function of piRNAs is the protection of the germline from the deleterious effects of mobile genetic elements such as transposons and viruses. This is accomplished via regulation of expression at two levels, during transcription and post-transcription, which will be discussed in greater detail later in this chapter.

Specialized functions of the piRNA pathway in vertebrates

Studies in mice have revealed that the most abundant piRNA species in the mammalian germ cells are pachytene piRNAs, so named as they arise during the pachytene stage of meiosis prophase I (Li, Roy et al. 2013). These piRNAs are involved in the maintenance of the male germline, though the mechanism of action remains to be characterized (Wu, Fu et al. 2020) Knockdown of *Miwi*, the homolog of *D. melanogaster Piwi* in mice, as well as loss of pachytene piRNA loci leads to male sterility (Zheng and Wang 2012, Wu, Pang et al. 2020).

Loss of function of PIWI-clade proteins in human cell culture has been associated with tumorigenesis. This points to a regulatory role for piRNAs in cancer development, which can be exploited for use as biomarkers for cancer diagnosis (Cammarata, de Miguel-Perez et al. 2022). In domestic chicken (*Gallus gallus*), infection with Avian leukosis virus (ALV) is

countered by an antiviral piRNA response from a recently endogenized defective provirus (Sun, Xie et al. 2017). This locus, which is highly expressed in the testes, was previously identified as producing an antiviral factor, and further analysis revealed the antiviral factor to be piRNAs. (Sun, Xie et al. 2017). Recently, the piRNA pathway was implicated in neural crest development, the first finding of somatic mammalian Piwi and piRNAs (Galton, Fejes-Toth et al. 2022).

Specialized functions of the piRNA pathway in invertebrates

piRNAs are widely expressed in arthropods and mollusks, with somatic expression of these small RNAs and a large variety of PIWI-clade proteins occurring in both phyla (Jehn, Gebert et al. 2018, Lewis, Quarles et al. 2018). In addition to defending the genome from TEs, some additional functions of the pathway have been identified. For example, in the silkworm *Bombyx mori*, the piRNA pathway is involved in sex determination through the activity of a feminizing piRNA (Kiuchi, Koga et al. 2014). In *Ae. aegypti*, additional functions of the piRNAs and associated PIWI proteins have been identified include gene regulation (Arensburger, Hice et al. 2011, Girardi, Miesen et al. 2017), maternal mRNA degradation during embryonic development (Halbach, Miesen et al. 2020), and endogenous viral element (EVE)-mediated immune memory to viruses infecting the ovaries (Suzuki, Baidaliuk et al. 2020).

## The piRNA pathway in Drosophila melanogaster

Research in *Drosophila melanogaster* has contributed greatly to our understanding of the piRNA pathway. The PIWI protein sub-family members expressed in the fruit fly are *Aubergine* (*Aub*), *Piwi* and *Argonaute-3* (*Ago3*) and their expression is limited mostly to the germline oocytes while Piwi is also expressed in the surrounding follicular cells (Brennecke, Aravin et al. 2007). In the germline cells, Aub and Ago3 proteins are found in the cytoplasm while Piwi is a nuclear protein. (Brennecke, Aravin et al. 2007, Nishida, Saito et al. 2007).

piRNA biogenesis has been best described in *D. melanogaster*. In the ovarian germ cells, piRNAs are mainly transcribed from dual-stranded piRNA clusters which are marked by the deposition of silencing histone modifications by Piwi on the clusters (Klattenhoff, Xi et al. 2009). Transcription from dual-stranded clusters occurs in both directions, starting from internal sites defined by the Rhino protein, and produces both sense and anti-sense piRNAs. RNA polymerase II is recruited to the locus by a complex formed by the Rhino, Deadlock and Cutoff (RDC) proteins (Mohn, Sienski et al. 2014, Andersen, Tirian et al. 2017). The primary transcripts vary in length and are further processed in the nucleus by the Cutoff protein, which protects the 5’ end of the transcript, preventing splicing or poly-A adenylation (Le Thomas, Stuwe et al. 2014). The primary piRNA transcripts are transported to the cytoplasm where further processing occurs in peri-nuclear structures known as nuage (Pane, Jiang et al. 2011, Zhang, Wang et al. 2012).

The nuage is a dense ribonucleoprotein cytoplasmic peri-nuclear granule found in germline cells which is a key organelle in the processing of RNA shuttled from the nucleus (Findlay 2003, Snee & Mcdonald 2004). In the nuage, the primary piRNAs which are mostly antisense to the transposon targets, are loaded onto Aub, and the Aub-piRISC then cleaves transposon mRNA (Malone, Brennecke et al. 2009). An RNA helicase protein, Vasa, facilitates unloading of the cleavage products from Aub, and loads the 3' fragment onto Ago3 (Xiol, Spinelli et al. 2014). The 3' end of the Ago3-bound secondary piRNA is processed to form a mature piRNA which is then loaded into the Ago3-piRISC, which can then cleave primary piRNAs for loading onto Aub (Hayashi, Schnabl et al. 2016). This continues in a cycle that leads to the amplification of the piRNA pool in the cell, known as the ping-pong amplification cycle.(Li et al 2009) The Ago3 and Aub associated piRNAs have a characteristic 10A and 1U bias respectively, as well as significant overlap in the first 10 nucleotides of the piRNAs due to the ping-pong cycle (Brennecke, Aravin et al. 2007, Gunawardane, Saito et al. 2007). In summary, Ago3 and Aub act post-transcriptionally to silence transposon expression by cleaving transposon mRNA. In the cytoplasm, Piwi is loaded with Ago3 cleavage products after processing by the Zucchini (Zuc) protein , an endonuclease on the outer mitochondrial membrane, which determines the 3' and 5' ends of the mature piRNAs in a process termed phased piRNA biogenesis (Wang, Han et al. 2015).

In the follicular cells surrounding the ovarian germ cells, Piwi is the only PIWI-clade protein expressed, therefore piRNAs are produced using the phased piRNA biogenesis pathway (Mohn, Handler et al. 2015). piRNA clusters in these cells are mostly uni-stranded and have promoters. Transcription therefore follows the canonical process involving RNA polymerase II and producing antisense piRNAs that are spliced, capped and poly-adenylated (Goriaux, Desset et al. 2014). The piRNA precursors are exported to the nucleus and undergo further processing in the Yb body which comprises of the proteins Yb and Armitage among others (Saito, Ishizu et al. 2010). WHAT DOES THE YB BODY DO? Armitage translocates the piRNA transcripts to the mitochondria, where proteins located on the outer mitochondria, Gasz and Daedalus unwind the piRNA from Armitage, allowing Zuc access to the 3’ and 5’ ends of the piRNAs resulting in the mature piRNA loaded onto Piwi forming the Piwi-piRISC. (Wang, Han et al. 2015).

The Piwi-piRISC is translocated to the nucleus where it is recruited to actively expressed transposons through recognition of primary transcripts as they are produced (Le Thomas, Rogers et al. 2013). Piwi induces silencing of the target locus at the transcription level by recruiting a histone H3K9 methyltransferase which deposits silencing modifications to the chromatin in the target and surrounding regions (Le Thomas, Stuwe et al. 2014). The region is further compacted into heterochromatin, preventing further expression from the locus (Andersen, Tirian et al. 2017).

## PART III: The piRNA pathway in *Aedes aegypti*

The yellow fever mosquito expresses an expanded repertoire of PIWI-clade proteins compared to *D. melanogaster*, with seven Piwi proteins and one Ago3 protein (Lewis, Salmela et al. 2016). Additionally, while piRNA and PIWI expression is mostly limited to the germline and supporting follicle cells in the fruit fly, piRNAs are expressed somatically in *Ae. aegypti* with developmental stage-specific expression (Miesen, Joosten et al. 2016). Piwi4-6 and Ago3 are expressed in somatic tissue and during larval and pupal development, Piwi7 is restricted to the developing embryo, while Piwi2 and Piwi3 are expressed in gonadal tissue of both male and female mosquitoes (Akbari, Antoshechkin et al. 2013).

Interestingly, the proportion of piRNAs mapping to transposable elements in *Ae. aegypti* is much lower (19%) than it is in *D. melanogaster* (50%), though transposable elements comprise approximately of the yellow fever mosquito genome compared to 3.5% in the fruit fly (Arensburger, Hice et al. 2011). This sparked interest in identifying the additional targets of genome-encoded piRNAs, with one such species being endogenous viral element (EVE)-derived piRNAs (Suzuki, Frangeul et al. 2017, Whitfield, Dolan et al. 2017, Aguiar, de Almeida et al. 2020). piRNA biogenesis in *Ae. aegypti* will be described in greater detail in Chapter 3.

Investigations in cell culture identified piRNAs produced in response to viral infection (vpiRNAs) (Miesen, Ivens et al. 2016, Varjak, Donald et al. 2017), which was confirmed in

infected mosquitoes (Marconcini, Pischedda et al. 2021), while similar studies in *D. melanogaster* did not indicate the presence of such vpiRNAs (Petit, Mongelli et al. 2016). The antiviral nature of vpiRNAs was confirmed by using CRISPR-Cas9 to delete a Cell Fusing Agent Virus (CFAV)-EVE (Suzuki, Baidaliuk et al. 2020). Infection with the cognate virus resulted in significantly increased CFAV replication and a higher viral load in the ovaries relative to "wild type" mosquitoes. (Suzuki, Baidaliuk et al. 2020).

The piRNA pathway serves as an adaptive immune system in *Aedes aegypti*.

Duplication of *piwi* genes in some dipterans has been shown to lead to functional specialization, strengthening the case that *D. melanogaster* may be somewhat unique in its germline constrained piRNAs and distinct Piwi and Aub proteins (Lewis, Salmela et al. 2016, Lewis, Quarles et al. 2018). The production of antiviral piRNAs in *Ae. aegypti* during active infection, the formation of viral DNA (vDNA) during viral infection, and the production of piRNAs from endogenized viruses, together present a picture of a heritable adaptive immune system in *Ae. aegypti*. The chance capture and endogenization of a viral fragment into the mosquito genome by TE activity, forms a new EVE, which then begins expressing piRNAs through a currently unknown mechanism (Goic, Stapleford et al. 2016, Ophinni, Palatini et al. 2019).The EVE would experience positive selection in the mosquito population, as it would confer heritable antiviral protection in addition to the siRNA pathway during active infection with the cognate virus. This system would provide immune

protection to the mosquito as it encounters a heavy viral challenge during two life stages: the voracious feeding in the aquatic larval stage and blood-feeding in the adult female. Altogether, this system prevents a novel opportunity for the control of arboviruses through the manipulation of *Ae. aegypti* "immunity" to viruses of medical and agricultural concern.

The expansion of the *piwi* genes associated with the piRNA pathway in *Ae. aegypti* along with the somatic expression of piRNAs in the mosquito raise questions as to what, if any, the additional roles of these genes may be in the mosquito. Experiments done both in cell culture as well as in the adult female mosquito pointed to the existence of vpiRNAs raised in response to acute infection and transcribed from EVE-containing loci. These EVEs represent a snapshot of the viruses encountered in *Ae. aegypti* evolutionary history in a particular region and are therefore mostly composed of insect specific viruses (ISVs), perhaps representing antiviral memory to locally circulating ISVs which are pathogenic to the insect.

The relatively recent confirmation of the antiviral nature of vpiRNAs in *Ae. aegypti* ovaries has raised important questions about the potential immune functions of the pathway in the yellow fever mosquito. Potential roles for the different Piwi proteins have been proposed using experiments in cell culture, however, we do not have tissue-specific confirmation of the presence of these proteins in the different developmental stages.

I aim to confirm the presence of each somatic Piwi protein (Piwi 4, Piwi5, Piwi6 and Ago3) in larval tissues which serve as barriers to viral transmission, specifically the midgut. This will

help to narrow down the proteins involved in RNA silencing in somatic tissues, which should be the same proteins involved in the response to infection with a virus pathogenic to mosquitoes.

My aim was to test this via the genetic transformation of *Ae. aegypti* with a segment of the SINV virus that produces a significant vpiRNA response both in cell culture and in infected mosquitoes. This will allow us to potentially elucidate the mechanism behind EVE-piRNA cluster formation, and testing a line of mosquitoes confirmed to express the SINV-piRNAs will allow for the confirmation of the antiviral nature of vpiRNAs in somatic tissues. I was successful in my aim to insert a plasmid into a complex highly repetitive locus, confirmed by fluorescence and sequencing.

The ability to generate even partial immunity to a virus of medical importance via gene editing would be a particularly promising tool to employ in the arsenal against mosquitoes and mosquito-borne diseases, as transmission of viruses from vertebrate to vertebrate depends on viral replication and accumulation in the salivary glands before the next bloodmeal.

BIBLIOGRAPHY

Adams, K. L., D. G. Abernathy, B. C. Willett, E. K. Selland, M. A. Itoe and F. Catteruccia (2021). "Wolbachia cifB induces cytoplasmic incompatibility in the malaria mosquito vector." Nat Microbiol **6**(12): 1575-1582.

Aguiar, E. R. G. R., J. P. P. de Almeida, L. R. Queiroz, L. S. Oliveira, R. P. Olmo, I. J. d. S. de Faria, J.-L. Imler, A. Gruber, B. J. Matthews and J. T. Marques (2020). "A single unidirectional piRNA cluster similar to the flamenco locus is the major source of EVE-derived transcription and small RNAs in Aedes aegypti mosquitoes." RNA **26**(5): 581-594.

Akbari, O. S., I. Antoshechkin, H. Amrhein, B. Williams, R. Diloreto, J. Sandler and B. A. Hay (2013). "The developmental transcriptome of the mosquito Aedes aegypti, an invasive species and major arbovirus vector." G3 **3**(9): 1493-1509.

Andersen, P. R., L. Tirian, M. Vunjak and J. Brennecke (2017). "A heterochromatin-dependent transcription machinery drives piRNA expression." Nature **549**(7670): 54-59.

Ant, T. H., C. S. Herd, V. Geoghegan, A. A. Hoffmann and S. P. Sinkins (2018). "The Wolbachia strain wAu provides highly efficient virus transmission blocking in Aedes aegypti." PLoS Pathog. **14**(1): e1006815.

Ant, T. H. and S. P. Sinkins (2018). "A Wolbachia triple-strain infection generates self-incompatibility in Aedes albopictus and transmission instability in Aedes aegypti." Parasit. Vectors **11**(1): 295.

Aravin, A., D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf, N. Iovino, P. Morris, M. J. Brownstein, S. Kuramochi-Miyagawa, T. Nakano, M. Chien, J. J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan and T. Tuschl (2006). "A novel class of small RNAs bind to MILI protein in mouse testes." Nature **442**(7099): 203-207.

Aravin, A. A., M. Lagos-Quintana, A. Yalcin, M. Zavolan, D. Marks, B. Snyder, T. Gaasterland, J. Meyer and T. Tuschl (2003). "The small RNA profile during Drosophila melanogaster development." Dev. Cell **5**(2): 337-350.

Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky and V. A. Gvozdev (2001). "Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline." Curr. Biol. **11**(13): 1017-1027.

Arensburger, P., R. H. Hice, J. A. Wright, N. L. Craig and P. W. Atkinson (2011). "The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs." BMC Genomics **12**: 606.

Asgari, S. (2014). "Role of microRNAs in arbovirus/vector interactions." Viruses **6**(9): 3514-3534.
Atwood, K. C., R. C. Von Borstel and A. R. Whiting (1956). "An Influence of Ploidy on the Time of Expression of Dominant Lethal Mutations in Habrobracon." Genetics **41**(6): 804-813.

Ayettey, J., A. Ablorde, G. K. Amlalo, B. A. Mensah and A. A. Kudom (2023). "Entomological surveillance on Aedes aegypti during covid 19 period in Cape Coast, Ghana: Risk of arboviral outbreaks, multiple insecticide resistance and distribution of F1534C, V410L and V1016I kdr mutations." PLoS Negl. Trop. Dis. **17**(5): e0011397.

Balaji, S., S. Jayachandran and S. R. Prabagaran (2019). "Evidence for the natural occurrence of Wolbachia in Aedes aegypti mosquitoes." FEMS Microbiol. Lett. **366**(6).

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Beckmann, J. F., J. A. Ronau and M. Hochstrasser (2017). "A Wolbachia deubiquitylating enzyme induces cytoplasmic incompatibility." Nat Microbiol **2**: 17007.

Blair, C. D. and K. E. Olson (2015). "The role of RNA interference (RNAi) in arbovirus-vector interactions." Viruses **7**(2): 820-843.

Bonneau, M., C. Atyame, M. Beji, F. Justy, M. Cohen-Gonsaud, M. Sicard and M. Weill (2018). "Culex pipiens crossing type diversity is governed by an amplified and polymorphic operon of Wolbachia." Nat. Commun. **9**(1): 319.

Bonning, B. C. and M.-C. Saleh (2021). "The Interplay Between Viruses and RNAi Pathways in Insects." Annu. Rev. Entomol. **66**: 61-79.

Bořkovec, A. B. (1976). "Control and Management of Insect Populations by Chemosterilants." Environ. Health Perspect. **14**: 103-107.

Breeland, S. G., G. M. Jeffery, C. S. Lofgren and D. E. Weidhaas (1974). "Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. I. Characteristics of the test site and the natural population." Am. J. Trop. Med. Hyg. **23**(2): 274-281.

Brennecke, J., A. A. Aravin, A. Stark, M. Dus, M. Kellis, R. Sachidanandam and G. J. Hannon (2007). "Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila." Cell **128**(6): 1089-1103.

Bushland, R. C. and D. E. Hopkins (1951). "Experiments with Screw-Worm Flies Sterilized by X-Rays." J. Econ. Entomol. **44**(5): 725-731.

Cabrera, M. and K. Jaffe (2007). "An aggregation pheromone modulates lekking behavior in the vector mosquito Aedes aegypti (Diptera: Culicidae)." J. Am. Mosq. Control Assoc. **23**(1): 1-10.

Cammarata, G., D. de Miguel-Perez, A. Russo, A. Peleg, V. Dolo, C. Rolfo and S. Taverna (2022). "Emerging noncoding RNAs contained in extracellular vesicles: rising stars as biomarkers in lung cancer liquid biopsy." Ther. Adv. Med. Oncol. **14**: 17588359221131229.

Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." Cell **136**(4): 642-655.

Carvalho, D. O., A. R. McKemey, L. Garziera, R. Lacroix, C. A. Donnelly, L. Alphey, A. Malavasi and M. L. Capurro (2015). "Suppression of a Field Population of Aedes aegypti in Brazil by Sustained Release of Transgenic Male Mosquitoes." PLoS Negl. Trop. Dis. **9**(7): e0003864.

Champer, J., R. Reeves, S. Y. Oh, C. Liu, J. Liu, A. G. Clark and P. W. Messer (2017). "Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations." PLoS Genet. **13**(7): e1006796.

Christophers, S. R. (1960). "Aedes aegypti (L.) the yellow fever mosquito: its life history, bionomics and structure." Aëdes aegypti (L. ) the Yellow Fever Mosquito: its Life History, Bionomics and Structure.

Crawford, J. E., J. M. Alves, W. J. Palmer, J. P. Day, M. Sylla, R. Ramasamy, S. N. Surendran, W. C. t. Black, A. Pain and F. M. Jiggins (2017). "Population genomics reveals that an anthropophilic population of Aedes aegypti mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector." BMC Biol. **15**(1): 16.

Crawford, J. E., D. W. Clarke, V. Criswell, M. Desnoyer, D. Cornel, B. Deegan, K. Gong, K. C. Hopkins, P. Howell, J. S. Hyde, J. Livni, C. Behling, R. Benza, W. Chen, K. L. Dobson, C. Eldershaw, D. Greeley, Y. Han, B. Hughes, E. Kakani, J. Karbowski, A. Kitchell, E. Lee, T. Lin, J. Liu, M. Lozano, W. MacDonald, J. W. Mains, M. Metlitz, S. N. Mitchell, D. Moore, J. R. Ohm, K. Parkes, A. Porshnikoff, C. Robuck, M. Sheridan, R. Sobecki, P. Smith, J. Stevenson, J. Sullivan, B. Wasson, A. M. Weakley, M. Wilhelm, J. Won, A. Yasunaga, W. C. Chan, J. Holeman, N. Snoad, L. Upson, T. Zha, S. L. Dobson, F. S. Mulligan, P. Massaro and B. J. White (2020). "Efficient production of male Wolbachia-infected Aedes aegypti mosquitoes enables large-scale suppression of wild populations." Nat. Biotechnol. **38**(4): 482-492.

Deehan, M., W. Lin, B. Blum, A. Emili and H. Frydman (2021). "Intracellular Density of Wolbachia Is Mediated by Host Autophagy and the Bacterial Cytoplasmic Incompatibility Gene cifB in a Cell Type-Dependent Manner in Drosophila melanogaster." MBio **12**(1).

Degner, E. C. and L. C. Harrington (2016). "Polyandry Depends on Postmating Time Interval in the Dengue Vector Aedes aegypti." Am. J. Trop. Med. Hyg. **94**(4): 780-785.

Deredec, A., A. Burt and H. C. J. Godfray (2008). "The population genetics of using homing endonuclease genes in vector and pest management." Genetics **179**(4): 2013-2026.

Derryberry, O. M. and F. E. Gartrell (1952). "Trends in malaria control program of the Tennessee Valley Authority." Am. J. Trop. Med. Hyg. **1**(3): 500-507.

Elissa, N., J. Mouchet, F. Riviere, J. Y. Meunier and K. Yao (1993). "Resistance of Anopheles gambiae s.s. to pyrethroids in Côte d'Ivoire." Ann. Soc. Belg. Med. Trop. **73**(4): 291-294.

Evans, B. R., P. Kotsakiozi, A. L. Costa-da-Silva, R. S. Ioshino, L. Garziera, M. C. Pedrosa, A. Malavasi, J. F. Virginio, M. L. Capurro and J. R. Powell (2019). "Transgenic Aedes aegypti Mosquitoes Transfer Genes into a Natural Population." Sci. Rep. **9**(1): 13047.

Fawaz, E. Y., S. A. Allan, U. R. Bernier, P. J. Obenauer and J. W. Diclaro, 2nd (2014). "Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of Aedes aegypti." J. Vector Ecol. **39**(2): 347-354.

Ferguson, N. M., D. T. H. Kien, H. Clapham, R. Aguas, V. T. Trung, T. N. B. Chau, J. Popovici, P. A. Ryan, S. L. O'Neill, E. A. McGraw, V. T. Long, L. T. Dui, H. L. Nguyen, N. V. V. Chau, B. Wills and C. P. Simmons (2015). "Modeling the impact on virus transmission of Wolbachia-mediated blocking of dengue virus infection of Aedes aegypti." Sci. Transl. Med. **7**(279): 279ra237.

Ferroni, E., T. Jefferson and G. Gachelin (2012). "Angelo Celli and research on the prevention of malaria in Italy a century ago." J. R. Soc. Med. **105**(1): 35-40.

Galton, R., K. Fejes-Toth and M. E. Bronner (2022). "Co-option of the piRNA pathway to regulate neural crest specification." Science Advances **8**(32): eabn1441.

Gan, S. J., Y. Q. Leong, M. F. H. Bin Barhanuddin, S. T. Wong, S. F. Wong, J. W. Mak and R. B. Ahmad (2021). "Dengue fever and insecticide resistance in Aedes mosquitoes in Southeast Asia: a review." Parasit. Vectors **14**(1): 315.

Gantz, V. M., N. Jasinskiene, O. Tatarenkova, A. Fazekas, V. M. Macias, E. Bier and A. A. James (2015). "Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi." Proc. Natl. Acad. Sci. U. S. A. **112**(49): E6736-6743.

Geoghegan, V., K. Stainton, S. M. Rainey, T. H. Ant, A. A. Dowle, T. Larson, S. Hester, P. D. Charles, B. Thomas and S. P. Sinkins (2017). "Perturbed cholesterol and vesicular trafficking associated with dengue blocking in Wolbachia-infected Aedes aegypti cells." Nat. Commun. **8**(1): 526.

Girardi, E., P. Miesen, B. Pennings, L. Frangeul, M.-C. Saleh and R. P. van Rij (2017). "Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in Aedes aegypti." Nucleic Acids Res. **45**(8): 4881-4892.

Gloria-Soria, A., T. G. Chiodo and J. R. Powell (2018). "Lack of Evidence for Natural Wolbachia Infections in Aedes aegypti (Diptera: Culicidae)." J. Med. Entomol. **55**(5): 1354-1356.

Goic, B., K. A. Stapleford, L. Frangeul, A. J. Doucet, V. Gausson, H. Blanc, N. Schemmel-Jofre, G. Cristofari, L. Lambrechts, M. Vignuzzi and M.-C. Saleh (2016). "Virus-derived DNA drives mosquito vector tolerance to arboviral infection." Nat. Commun. **7**: 12410.

Goriaux, C., S. Desset, Y. Renaud, C. Vaury and E. Brasset (2014). "Transcriptional properties and splicing of the flamenco piRNA cluster." EMBO Rep. **15**(4): 411-418.

Gunawardane, L. S., K. Saito, K. M. Nishida, K. Miyoshi, Y. Kawamura, T. Nagami, H. Siomi and M. C. Siomi (2007). "A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila." Science **315**(5818): 1587-1590.

Gwadz, R. W., G. B. Craig, Jr. and W. A. Hickey (1971). "Female sexual behavior as the mechanism rendering Aedes aegypti refractory to insemination." Biol. Bull. **140**(2): 201-214.

Halbach, R., P. Miesen, J. Joosten, E. Taşköprü, I. Rondeel, B. Pennings, C. B. F. Vogels, S. H. Merkling, C. J. Koenraadt, L. Lambrechts and R. P. van Rij (2020). "A satellite repeat-derived piRNA controls embryonic development of Aedes." Nature.

Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, A. Burt, N. Windbichler, A. Crisanti and T. Nolan (2016). "A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae." Nat. Biotechnol. **34**(1): 78-83.

Hammond, A., P. Pollegioni, T. Persampieri, A. North, R. Minuz, A. Trusso, A. Bucci, K. Kyrou, I. Morianou, A. Simoni, T. Nolan, R. Müller and A. Crisanti (2021). "Gene-drive suppression of mosquito populations in large cages as a bridge between lab and field." Nat. Commun. **12**(1): 4589.

Hammond, A. M., K. Kyrou, M. Bruttini, A. North, R. Galizi, X. Karlsson, N. Kranjc, F. M. Carpi, R. D'Aurizio, A. Crisanti and T. Nolan (2017). "The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito." PLoS Genet. **13**(10): e1007039.

Harris, A. F., A. R. McKemey, D. Nimmo, Z. Curtis, I. Black, S. A. Morgan, M. N. Oviedo, R. Lacroix, N. Naish, N. I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C. A. Donnelly, W. D. Petrie and L. Alphey (2012). "Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes." Nat. Biotechnol. **30**(9): 828-830.

Harris, A. F., D. Nimmo, A. R. McKemey, N. Kelly, S. Scaife, C. A. Donnelly, C. Beech, W. D. Petrie and L. Alphey (2011). "Field performance of engineered male mosquitoes." Nat. Biotechnol. **29**(11): 1034-1037.

Hartberg, W. K. (1971). "Observations on the mating behaviour of Aedes aegypti in nature." Bull. World Health Organ. **45**(6): 847-850.

Hayashi, R., J. Schnabl, D. Handler, F. Mohn, S. L. Ameres and J. Brennecke (2016). "Genetic and mechanistic diversity of piRNA 3'-end formation." Nature **539**(7630): 588-592.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill and K. N. Johnson (2008). "Wolbachia and virus protection in insects." Science **322**(5902): 702.

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, H. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli and S. L. O'Neill (2011). "Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission." Nature **476**(7361): 454-457.

Hussain, M., S. Torres, E. Schnettler, A. Funk, A. Grundhoff, G. P. Pijlman, A. A. Khromykh and S. Asgari (2012). "West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells." Nucleic Acids Res. **40**(5): 2210-2223.

Jangir, P. K. and A. Prasad (2022). "Spatial distribution of insecticide resistance and susceptibility in Aedes aegypti and Aedes albopictus in India." Int. J. Trop. Insect Sci. **42**(2): 1019-1044.

Jehn, J., D. Gebert, F. Pipilescu, S. Stern, J. S. T. Kiefer, C. Hewel and D. Rosenkranz (2018). "PIWI genes and piRNAs are ubiquitously expressed in mollusks and show patterns of lineage-specific adaptation." Commun Biol **1**: 137.

Kandul, N. P., J. Liu, J. B. Bennett, J. M. Marshall and O. S. Akbari (2021). "A confinable home-and-rescue gene drive for population modification." Elife **10**.

Kandul, N. P., J. Liu, H. M. Sanchez C, S. L. Wu, J. M. Marshall and O. S. Akbari (2019). "Transforming insect population control with precision guided sterile males with demonstration in flies." Nat. Commun. **10**(1): 84.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat. Rev. Mol. Cell Biol. **10**(2): 126-139.

Kittayapong, P. (2021). Combined sterile insect technique and incompatible insect technique: concept, study design, experience and lessons learned from a pilot suppression trial in Thailand. Area-Wide Integrated Pest Management, CRC Press**:** 405-432.

Kittayapong, P., S. Ninphanomchai, W. Limohpasmanee, C. Chansang, U. Chansang and P. Mongkalangoon (2019). "Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress Aedes aegypti vector populations in semi-rural settings in Thailand." PLoS Negl. Trop. Dis. **13**(10): e0007771.

Kiuchi, T., H. Koga, M. Kawamoto, K. Shoji, H. Sakai, Y. Arai, G. Ishihara, S. Kawaoka, S. Sugano, T. Shimada, Y. Suzuki, M. G. Suzuki and S. Katsuma (2014). "A single female-specific piRNA is the primary determiner of sex in the silkworm." Nature **509**(7502): 633-636.

Klattenhoff, C., H. Xi, C. Li, S. Lee, J. Xu, J. S. Khurana, F. Zhang, N. Schultz, B. S. Koppetsch, A. Nowosielska, H. Seitz, P. D. Zamore, Z. Weng and W. E. Theurkauf (2009). "The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters." Cell **138**(6): 1137-1149.

Knipling, E. F. (1979). The Basic Principles of Insect Population Suppression and Management, U.S. Department of Agriculture.

Kyrou, K., A. M. Hammond, R. Galizi, N. Kranjc, A. Burt, A. K. Beaghton, T. Nolan and A. Crisanti (2018). "A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes." Nat. Biotechnol. **36**(11): 1062-1066.
Laven, H., J. Cousserans and G. Guille (1972). "Eradicating mosquitoes using translocations: a first field experiment." Nature **236**(5348): 456-457.

Le Thomas, A., A. K. Rogers, A. Webster, G. K. Marinov, S. E. Liao, E. M. Perkins, J. K. Hur, A. A. Aravin and K. F. Tóth (2013). "Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state." Genes Dev. **27**(4): 390-399.

Le Thomas, A., E. Stuwe, S. Li, J. Du, G. Marinov, N. Rozhkov, Y.-C. A. Chen, Y. Luo, R. Sachidanandam, K. F. Toth, D. Patel and A. A. Aravin (2014). "Transgenerationally inherited piRNAs trigger piRNA biogenesis by changing the chromatin of piRNA clusters and inducing precursor processing." Genes Dev. **28**(15): 1667-1680.

Lee, Y., H. Schmidt, T. C. Collier, W. R. Conner, M. J. Hanemaaijer, M. Slatkin, J. M. Marshall, J. C. Chiu, C. T. Smartt, G. C. Lanzaro, F. S. Mulligan and A. J. Cornel (2019). "Genome-wide divergence among invasive populations of Aedes aegypti in California." BMC Genomics **20**(1): 204.

Leftwich, P. T., L. G. Spurgin, T. Harvey-Samuel, C. J. E. Thomas, L. C. Paladino, M. P. Edgington and L. Alphey (2021). "Genetic pest management and the background genetics of release strains." Philos. Trans. R. Soc. Lond. B Biol. Sci. **376**(1818): 20190805.

LePage, D. P., J. A. Metcalf, S. R. Bordenstein, J. On, J. I. Perlmutter, J. D. Shropshire, E. M. Layton, L. J. Funkhouser-Jones, J. F. Beckmann and S. R. Bordenstein (2017). "Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility." Nature **543**(7644): 243-247.

Lewis, S. H., K. A. Quarles, Y. Yang, M. Tanguy, L. Frézal, S. A. Smith, P. P. Sharma, R. Cordaux, C. Gilbert, I. Giraud, D. H. Collins, P. D. Zamore, E. A. Miska, P. Sarkies and F. M. Jiggins (2018). "Pan-arthropod analysis reveals somatic piRNAs as an ancestral defence against transposable elements." Nat Ecol Evol **2**(1): 174-181.

Lewis, S. H., H. Salmela and D. J. Obbard (2016). "Duplication and Diversification of Dipteran Argonaute Genes, and the Evolutionary Divergence of Piwi and Aubergine." Genome Biol. Evol. **8**(3): 507-518.

Li, M., T. Yang, M. Bui, S. Gamez, T. Wise, N. P. Kandul, J. Liu, L. Alcantara, H. Lee, J. R. Edula, R. Raban, Y. Zhan, Y. Wang, N. DeBeaubien, J. Chen, H. M. Sánchez C, J. B. Bennett, I. Antoshechkin, C. Montell, J. M. Marshall and O. S. Akbari (2021). "Suppressing mosquito populations with precision guided sterile males." Nat. Commun. **12**(1): 5374.

Li, M., T. Yang, N. P. Kandul, M. Bui, S. Gamez, R. Raban, J. Bennett, H. M. Sánchez C, G. C. Lanzaro, H. Schmidt, Y. Lee, J. M. Marshall and O. S. Akbari (2020). "Development of a confinable gene drive system in the human disease vector Aedes aegypti." Elife **9**.

Li, X. Z., C. K. Roy, X. Dong, E. Bolcun-Filas, J. Wang, B. W. Han, J. Xu, M. J. Moore, J. C. Schimenti, Z. Weng and P. D. Zamore (2013). "An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes." Mol. Cell **50**(1): 67-81.

Lindquist, D. A., M. Abusowa and M. J. Hall (1992). "The New World screwworm fly in Libya: a review of its introduction and eradication." Med. Vet. Entomol. **6**(1): 2-8.

Lounibos, L. P. (1981). "Habitat segregation among African treehole mosquitoes." Ecol. Entomol. **6**(2): 129-154.

Malone, C. D., J. Brennecke, M. Dus, A. Stark, W. R. McCombie, R. Sachidanandam and G. J. Hannon (2009). "Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary." Cell **137**(3): 522-535.

Marconcini, M., E. Pischedda, V. Houé, U. Palatini, N. Lozada-Chávez, D. Sogliani, A.-B. Failloux and M. Bonizzoni (2021). "Profile of Small RNAs, vDNA Forms and Viral Integrations in Late Chikungunya Virus Infection of Aedes albopictus Mosquitoes." Viruses **13**(4): 553.

Messina, J. P., O. J. Brady, N. Golding, M. U. G. Kraemer, G. R. W. Wint, S. E. Ray, D. M. Pigott, F. M. Shearer, K. Johnson, L. Earl, L. B. Marczak, S. Shirude, N. Davis Weaver, M. Gilbert, R. Velayudhan, P. Jones, T. Jaenisch, T. W. Scott, R. C. Reiner, Jr. and S. I. Hay (2019). "The current and future global distribution and population at risk of dengue." Nat Microbiol **4**(9): 1508-1515.

Metzger, M. E., M. Hardstone Yoshimizu, K. A. Padgett, R. Hu and V. L. Kramer (2017). "Detection and Establishment of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) Mosquitoes in California, 2011-2015." J. Med. Entomol. **54**(3): 533-543.

Miesen, P., A. Ivens, A. H. Buck and R. P. van Rij (2016). "Small RNA Profiling in Dengue Virus 2-Infected Aedes Mosquito Cells Reveals Viral piRNAs and Novel Host miRNAs." PLoS Negl. Trop. Dis. **10**(2): e0004452.

Miesen, P., J. Joosten and R. P. van Rij (2016). "PIWIs Go Viral: Arbovirus-Derived piRNAs in Vector Mosquitoes." PLoS Pathog. **12**(12): e1006017.

Mohn, F., D. Handler and J. Brennecke (2015). "Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis." Science **348**(6236): 812-817.

Mohn, F., G. Sienski, D. Handler and J. Brennecke (2014). "The rhino-deadlock-cutoff complex licenses noncanonical transcription of dual-strand piRNA clusters in Drosophila." Cell **157**(6): 1364-1379.

Moyes, C. L., J. Vontas, A. J. Martins, L. C. Ng, S. Y. Koou, I. Dusfour, K. Raghavendra, J. Pinto, V. Corbel, J.-P. David and D. Weetman (2017). "Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans." PLoS Negl. Trop. Dis. **11**(7): e0005625.

Nájera, J. A., M. González-Silva and P. L. Alonso (2011). "Some lessons for the future from the Global Malaria Eradication Programme (1955-1969)." PLoS Med. **8**(1): e1000412.

Navarro-Payá, D., I. Flis, M. A. E. Anderson, P. Hawes, M. Li, O. S. Akbari, S. Basu and L. Alphey (2020). "Targeting female flight for genetic control of mosquitoes." PLoS Negl. Trop. Dis. **14**(12): e0008876.

Ngugi, H. N., F. M. Mutuku, B. A. Ndenga, P. S. Musunzaji, J. O. Mbakaya, P. Aswani, L. W. Irungu, D. Mukoko, J. Vulule, U. Kitron and A. D. LaBeaud (2017). "Characterization and productivity profiles of Aedes aegypti (L.) breeding habitats across rural and urban landscapes in western and coastal Kenya." Parasit. Vectors **10**(1): 331.

Nishida, K. M., K. Saito, T. Mori, Y. Kawamura, T. Nagami-Okada, S. Inagaki, H. Siomi and M. C. Siomi (2007). "Gene silencing mechanisms mediated by Aubergine piRNA complexes in Drosophila male gonad." RNA **13**(11): 1911-1922.

Nosrat, C., J. Altamirano, A. Anyamba, J. M. Caldwell, R. Damoah, F. Mutuku, B. Ndenga and A. D. LaBeaud (2021). "Impact of recent climate extremes on mosquito-borne disease transmission in Kenya." PLoS Negl. Trop. Dis. **15**(3): e0009182.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin and S. L. Dobson (2012). "Open release of male mosquitoes infected with a wolbachia biopesticide: field performance and infection containment." PLoS Negl. Trop. Dis. **6**(11): e1797.

Ophinni, Y., U. Palatini, Y. Hayashi and N. F. Parrish (2019). "piRNA-Guided CRISPR-like Immunity in Eukaryotes." Trends Immunol. **40**(11): 998-1010.

Organization, W. H. (1964). "Genetics of Vectors and Insecticide Resistance: Report of a WHO Scientific Group [meeting held in Geneva from 5 to 9 August 1963]."

Ouédraogo, W. M., K. H. Toé, A. Sombié, M. Viana, C. Bougouma, A. Sanon, D. Weetman, P. J. McCall, H. Kanuka and A. Badolo (2022). "Impact of physicochemical parameters of Aedes aegypti breeding habitats on mosquito productivity and the size of emerged adult mosquitoes in Ouagadougou City, Burkina Faso." Parasit. Vectors **15**(1): 478.

Pane, A., P. Jiang, D. Y. Zhao, M. Singh and T. Schüpbach (2011). "The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline." EMBO J. **30**(22): 4601-4615.

Petit, M., V. Mongelli, L. Frangeul, H. Blanc, F. Jiggins and M.-C. Saleh (2016). "piRNA pathway is not required for antiviral defense in Drosophila melanogaster." Proc. Natl. Acad. Sci. U. S. A. **113**(29): E4218-4227.

Phuc, H. K., M. H. Andreasen, R. S. Burton, C. Vass, M. J. Epton, G. Pape, G. Fu, K. C. Condon, S. Scaife, C. A. Donnelly, P. G. Coleman, H. White-Cooper and L. Alphey (2007). "Late-acting dominant lethal genetic systems and mosquito control." BMC Biol. **5**: 11.

Pless, E., A. Gloria-Soria, B. R. Evans, V. Kramer, B. G. Bolling, W. J. Tabachnick and J. R. Powell (2017). "Multiple introductions of the dengue vector, Aedes aegypti, into California." PLoS Negl. Trop. Dis. **11**(8): e0005718.

Powell, J. R. and W. J. Tabachnick (2013). "History of domestication and spread of Aedes aegypti--a review." Mem. Inst. Oswaldo Cruz **108 Suppl 1**: 11-17.

Price, T. A. R., N. Windbichler, R. L. Unckless, A. Sutter, J.-N. Runge, P. A. Ross, A. Pomiankowski, N. L. Nuckolls, C. Montchamp-Moreau, N. Mideo, O. Y. Martin, A. Manser, M. Legros, A. M. Larracuente, L. Holman, J. Godwin, N. Gemmell, C. Courret, A. Buchman, L. G. Barrett and A. K. Lindholm (2020). "Resistance to natural and synthetic gene drive systems." J. Evol. Biol. **33**(10): 1345-1360.

Robine, N., N. C. Lau, S. Balla, Z. Jin, K. Okamura, S. Kuramochi-Miyagawa, M. D. Blower and E. C. Lai (2009). "A Broadly Conserved Pathway Generates 3′UTR-Directed Primary piRNAs." Curr. Biol. **19**(24): 2066-2076.

Robinson, A. S. (2005). "Genetic basis of the sterile insect technique." Sterile insect technique: principles and practice in area.

Rose, N. H., A. Badolo, M. Sylla, J. Akorli, S. Otoo, A. Gloria-Soria, J. R. Powell, B. J. White, J. E. Crawford and C. S. McBride (2023). "Dating the origin and spread of specialization on human hosts in Aedes aegypti mosquitoes." Elife **12**.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault and M. Solignac (1997). "Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods." Proceedings of the Royal Society of London. Series B: Biological Sciences **250**(1328): 91-98.

Ryan, P. A., A. P. Turley, G. Wilson, T. P. Hurst, K. Retzki, J. Brown-Kenyon, L. Hodgson, N. Kenny, H. Cook, B. L. Montgomery, C. J. Paton, S. A. Ritchie, A. A. Hoffmann, N. P. Jewell, S. K. Tanamas, K. L. Anders, C. P. Simmons and S. L. O'Neill (2019). "Establishment of wMel Wolbachia in Aedes aegypti mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia." Gates Open Res **3**: 1547.

Ryan, S. J., C. J. Carlson, E. A. Mordecai and L. R. Johnson (2019). "Global expansion and redistribution of Aedes-borne virus transmission risk with climate change." PLoS Negl. Trop. Dis. **13**(3): e0007213.

Saito, K., S. Inagaki, T. Mituyama, Y. Kawamura, Y. Ono, E. Sakota, H. Kotani, K. Asai, H. Siomi and M. C. Siomi (2009). "A regulatory circuit for piwi by the large Maf gene traffic jam in Drosophila." Nature **461**(7268): 1296-1299.

Saito, K., H. Ishizu, M. Komai, H. Kotani, Y. Kawamura, K. M. Nishida, H. Siomi and M. C. Siomi (2010). "Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in Drosophila." Genes Dev. **24**(22): 2493-2498.

Saito, K., Y. Sakaguchi, T. Suzuki, T. Suzuki, H. Siomi and M. C. Siomi (2007). "Pimet, the Drosophila homolog of HEN1, mediates 2'-O-methylation of Piwi- interacting RNAs at their 3' ends." Genes Dev. **21**(13): 1603-1608.

Schmidt, T. L., N. H. Barton, G. Rašić, A. P. Turley, B. L. Montgomery, I. Iturbe-Ormaetxe, P. E. Cook, P. A. Ryan, S. A. Ritchie, A. A. Hoffmann, S. L. O'Neill and M. Turelli (2017). "Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti." PLoS Biol. **15**(5): e2001894.

Scott, T. W., G. G. Clark, L. H. Lorenz, P. H. Amerasinghe, P. Reiter and J. D. Edman (1993). "Detection of multiple blood feeding in Aedes aegypti (Diptera: Culicidae) during a single gonotrophic cycle using a histologic technique." J. Med. Entomol. **30**(1): 94-99.

Servick, K. (2019). "GM mosquito study draws fire." Science **365**(6459): 1234.

Shanahan, T. M., N. P. McKay, K. A. Hughen, J. T. Overpeck, B. Otto-Bliesner, C. W. Heil, J. King, C. A. Scholz and J. Peck (2015). "The time-transgressive termination of the African Humid Period." Nat. Geosci. **8**(2): 140-144.

Shousha, A. T. (1948). "Species-eradication: The Eradication of Anopheles gambioe from Upper Egypt, 1942-1945." Bull. World Health Organ. **1**(2): 309-352.

Smith, R. H. and R. C. von Borstel (1972). "Genetic control of insect populations." Science **178**(4066): 1164-1174.

Sudharshan, S., R. Naidu, M. Mallavarapu and N. Bolan (2012). "DDT remediation in contaminated soils: a review of recent studies." Biodegradation **23**(6): 851-863.

Sun, Y. H., L. H. Xie, X. Zhuo, Q. Chen, D. Ghoneim, B. Zhang, J. Jagne, C. Yang and X. Z. Li (2017). "Domestic chickens activate a piRNA defense against avian leukosis virus." Elife **6**.

Suzuki, Y., A. Baidaliuk, P. Miesen, L. Frangeul, A. B. Crist, S. H. Merkling, A. Fontaine, S. Lequime, I. Moltini-Conclois, H. Blanc, R. P. van Rij, L. Lambrechts and M.-C. Saleh (2020). "Non-retroviral endogenous viral element limits cognate virus replication in Aedes aegypti ovaries." Curr. Biol.

Suzuki, Y., L. Frangeul, L. B. Dickson, H. Blanc, Y. Verdier, J. Vinh, L. Lambrechts and M.-C. Saleh (2017). "Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes." J. Virol. **91**(15).

Tabachnick, W. J. (1991). "Evolutionary Genetics and Arthropod-borne Disease: The Yellow Fever Mosquito." Am. Entomol. **37**(1): 14-26.

Takken, W., W. B. Snellen, J. P. Verhave, B. G. J. Knols and S. Atmosoedjono (1990). "Environmental measures for malaria control in Indonesia -an historical review on species sanitation." WAGENINGEN AGRICULTURAL UNIVERSITY PAPERS.

Tungu, P., B. Kabula, T. Nkya, P. Machafuko, E. Sambu, B. Batengana, W. Sudi, Y. A. Derua, V. Mwingira, D. Masue, R. Malima, C. Kitojo, N. Serbantez, E. J. Reaves, C. Mwalimu, S. L. Nhiga, M. Ally, H. R. Mkali, J. J. Joseph, A. Chan, J. Ngondi, S. Lalji, S. Nyinondi, E. Eckert, R. Reithinger, S. Magesa and W. N. Kisinza (2023). "Trends of insecticide resistance monitoring in mainland Tanzania, 2004-2020." Malar. J. **22**(1): 100.

Unckless, R. L., A. G. Clark and P. W. Messer (2017). "Evolution of Resistance Against CRISPR/Cas9 Gene Drive." Genetics **205**(2): 827-841.

Vagin, V. V., A. Sigova, C. Li, H. Seitz, V. Gvozdev and P. D. Zamore (2006). "A distinct small RNA pathway silences selfish genetic elements in the germline." Science **313**(5785): 320-324.

van den Berg, H., H. S. da Silva Bezerra, S. Al-Eryani, E. Chanda, B. N. Nagpal, T. B. Knox, R. Velayudhan and R. S. Yadav (2021). "Recent trends in global insecticide use for disease vector control and potential implications for resistance management." Sci. Rep. **11**(1): 23867.

van Rij, R. P., M.-C. Saleh, B. Berry, C. Foo, A. Houk, C. Antoniewski and R. Andino (2006). "The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster." Genes Dev. **20**(21): 2985-2995.

Varjak, M., C. L. Donald, T. J. Mottram, V. B. Sreenu, A. Merits, K. Maringer, E. Schnettler and A. Kohl (2017). "Characterization of the Zika virus induced small RNA response in Aedes aegypti cells." PLoS Negl. Trop. Dis. **11**(10): e0006010.

Wang, W., B. W. Han, C. Tipping, D. T. Ge, Z. Zhang, Z. Weng and P. D. Zamore (2015). "Slicing and Binding by Ago3 or Aub Trigger Piwi-Bound piRNA Production by Distinct Mechanisms." Mol. Cell **59**(5): 819-830.

Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed and J. J. Welch (2015). "The incidence of bacterial endosymbionts in terrestrial arthropods." Proc. Biol. Sci. **282**(1807): 20150249.

Werren, J. H., L. Baldo and M. E. Clark (2008). "Wolbachia: master manipulators of invertebrate biology." Nat. Rev. Microbiol. **6**(10): 741-751.

Whitfield, Z. J., P. T. Dolan, M. Kunitomi, M. Tassetto, M. G. Seetin, S. Oh, C. Heiner, E. Paxinos and R. Andino (2017). "The Diversity, Structure, and Function of Heritable Adaptive Immunity Sequences in the Aedes aegypti Genome." Curr. Biol. **27**(22): 3511-3519.e3517.

Wilson, A. L., O. Courtenay, L. A. Kelly-Hope, T. W. Scott, W. Takken, S. J. Torr and S. W. Lindsay (2020). "The importance of vector control for the control and elimination of vector-borne diseases." PLoS Negl. Trop. Dis. **14**(1): e0007831.

Wu, H., R. Pang, T. Cheng, L. Xue, H. Zeng, T. Lei, M. Chen, S. Wu, Y. Ding, J. Zhang, M. Shi and Q. Wu (2020). "Abundant and Diverse RNA Viruses in Insects Revealed by RNA-Seq Analysis: Ecological and Evolutionary Implications." mSystems **5**(4).

Wu, P.-H., Y. Fu, K. Cecchini, D. M. Özata, A. Arif, T. Yu, C. Colpan, I. Gainetdinov, Z. Weng and P. D. Zamore (2020). "The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility." Nat. Genet. **52**(7): 728-739.

Xiol, J., P. Spinelli, M. A. Laussmann, D. Homolka, Z. Yang, E. Cora, Y. Couté, S. Conn, J. Kadlec, R. Sachidanandam, M. Kaksonen, S. Cusack, A. Ephrussi and R. S. Pillai (2014). "RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts." Cell **157**(7): 1698-1711.
Yadav, A. K., C. Butler, A. Yamamoto, A. A. Patil, A. L. Lloyd and M. J. Scott (2023).

"CRISPR/Cas9-based split homing gene drive targeting doublesex for population suppression of the global fruit pest Drosophila suzukii." Proc. Natl. Acad. Sci. U. S. A. **120**(25): e2301525120.
Yao, F. A., A.-A. Millogo, P. S. Epopa, A. North, F. Noulin, K. Dao, M. Drabo, C. Guissou, S. Kekele, M. Namountougou, R. K. Ouedraogo, L. Pare, N. Barry, R. Sanou, H. Wandaogo, R. K. Dabire, A. McKemey, F. Tripet and A. Diabaté (2022). "Mark-release-recapture experiment in Burkina Faso demonstrates reduced fitness and dispersal of genetically-modified sterile malaria mosquitoes." Nat. Commun. **13**(1): 796.

Yen, J. H. and A. R. Barr (1973). "The etiological agent of cytoplasmic incompatibility in Culex pipiens." J. Invertebr. Pathol. **22**(2): 242-250.

Zhang, F., J. Wang, J. Xu, Z. Zhang, B. S. Koppetsch, N. Schultz, T. Vreven, C. Meignin, I. Davis, P. D. Zamore, Z. Weng and W. E. Theurkauf (2012). "UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery." Cell **151**(4): 871-884.

Zheng, K. and P. J. Wang (2012). "Blockade of pachytene piRNA biogenesis reveals a novel requirement for maintaining post-meiotic germline genome integrity." PLoS Genet. **8**(11): e1003038.

Zheng, X., D. Zhang, Y. Li, C. Yang, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, J. Bouyer, M. Tang, B. Zheng, J. Yu, J. Liu, J. Zhuang, Z. Hu, M. Zhang, J.-T. Gong, X.-Y. Hong, Z. Zhang, L. Lin, Q. Liu, Z. Hu, Z. Wu, L. A. Baton, A. A. Hoffmann and Z. Xi (2019). "Incompatible and sterile insect techniques combined eliminate mosquitoes." Nature **572**(7767): 56-61.

| Vector control method | | Pros | Cons | Proposed modifications | References |
|---|---|---|---|---|---|
| SIT | Chemical | Relatively cheap | Migration of wild-type males; handling of toxic chemicals | | Gato et al 2014 |
| | Irradiation | Any females accidentally released are sterile | Migration; differences in radiosensitivity in different insects therefore the sterilizing dose needs to be optimized; irradiation reduces fitness | | LaChance & Graham 1984; Oliva et al 2012; Gato et al 2021; Yamada et al 2023; |
| | Wolbachia for population suppression | Migration does not overwhelm suppression | Accidental release of females – establish a new colony; large-scale rearing of infected males is expensive | Automation of rearing & sex sorting; pupal irradiation; temperature-lethal female strain | Crawford et al 2020; Zheng et al 2019; Kittayapong et al 2019 Ndo et al 2018 |
| | RIDL | Late-acting lethality | Introgression of the introduced genome | | Phuc et al 2007; Evans et al 2019; |
| Chemical insecticides | | Relatively cheap and easy to deploy | Resistance develops rapidly | | Yang F et al 2020; Kasai S et al 2022 |
| Gene drive | | Super-Mendelian inheritance rates; | Release of GMOs; development of resistance alleles; migration | Double-tap gene drive; genetic sexing strains | Reid W et al 2022; Bishop et al 2022; Lutrat et al 2023 |
| Biological control | Larval stage control | Cheap; complements chemical insecticides | Unreliable | | Pyke GH 2008 |

**Table 1.1:** A summary of the main vector control methods currently in use, their pros, and cons, as well as any proposed modifications to the technology.

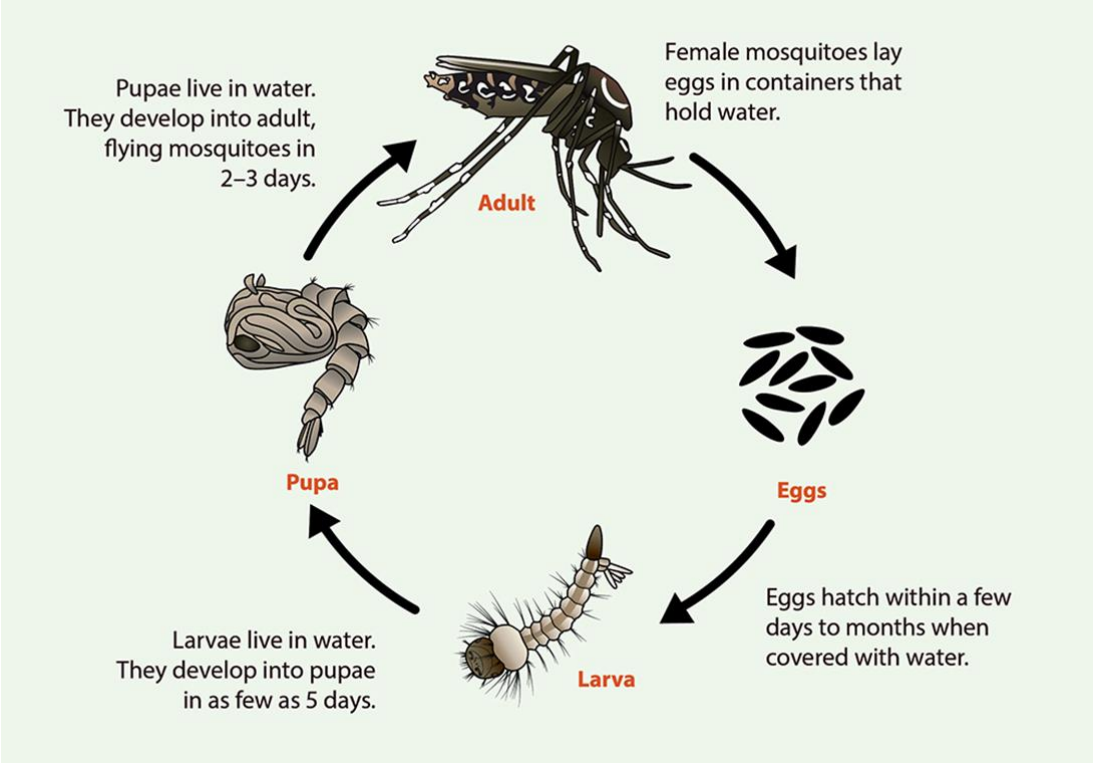


**Figure 1.1:** The life cycle of the mosquito *Aedes aegypti*, adapted from (CDC 2022).

# CHAPTER TWO

## Using CRISPR-Cas9 to investigate the function of the expanded PIWI repertoire in *Aedes aegypti.*

## INTRODUCTION

### PIWI structure

Argonaute proteins can be divided into two main clades: AGO and PIWI-clade proteins, with a third clade found only in nematodes known as WAGO (Worm-specific Argonautes). These proteins are found in all eukaryotes, except *Saccharomyces cerevisiae,* as well as some prokaryotes, and are important in the formation of RNA-induced silencing complexes (RISCs), which are the main effectors of RNA-guided gene expression regulation (Hammond, Bernstein et al. 2000, Drinnenberg, Weinberg et al. 2009). The RNA cleavage function of Argonautes was first confirmed by mutational analysis of mammalian Ago2 (Liu, Carmell et al. 2004).

AGO-clade proteins bind 20-22 nucleotide long small RNAs, mostly siRNAs and miRNAs, while PIWI-clade proteins bind slightly longer (23-30 nucleotide) piRNAs. (Meister 2013). X-ray crystallography confirmed that the PIWI domain in Argonaute proteins adopts a ribonuclease-H (RNase-H) fold, a characteristic tertiary protein structure which brings the active site residues DEDX, (Aspartate-Glutamate-Aspartate-usually Histidine) together to

cleave the target when present, catalyzed by a divalent metal ion. (Yang and Steitz 1995, Chapados, Chai et al. 2001, Song, Smith et al. 2004, Rivas, Tolia et al. 2005, Nakanishi, Weinberg et al. 2012).

The PAZ domain is named after three proteins that contain this domain, that is, Piwi, Argonaute and Zwille/Pinhead proteins (Cerutti, Mian et al. 2000). It consists of two sub-domains which form a fold similar to that found in single-stranded nucleic acid binding proteins known as the oligonucleotide/oligosaccharide binding-fold (OB-fold) and is involved in the binding of ssRNAs (Murzin 1993, Song, Liu et al. 2003). The main function of the PAZ domain is recognizing the 3' overhang terminal of ssRNAs generated by processing by other RNAse-H like proteins such as Dicer, allowing for selective binding that precludes recognition of self RNA degradation products (Song, Liu et al. 2003).

The PIWI domain has been found to adopt an RNase-H-like fold that catalyzes the cleavage of RNA using a nucleic acid template (Cerutti, Mian et al. 2000, Yuan, Pei et al. 2005). Like other proteins with RNase-H type activity, cleavage leads to the production of a 3'-hydroxyl and 5'-phosphate on the RNA product, with the catalytic motif positioned proximal to the scissile phosphate on the target RNA between the tenth and eleventh nucleotides from the 5' end of the "guide" strand. (Elbashir, Lendeckel et al. 2001, Liu, Carmell et al. 2004, Song, Smith et al. 2004)

The "middle" domain, also known as Mid domain, contains a highly conserved pocket-like structure that is lined with mostly basic amino acid residues and is involved in anchoring the 5'-phosphate of the bound ssRNA molecule (Parker, Roe et al. 2005, Yuan, Pei et al. 2005).

Together, the N terminal, Mid and PIWI domains form a bowl-shaped structure with the PIWI domain in the center, with the PAZ domain closing off the top of the structure like a lid (Song, Smith et al. 2004). The bound RNA guide sits partially within the bowl, with the 3' end bound to PAZ and the 5' end nestled in the Mid domain where the RNA is then anchored and the 3' end is released allowing for base-pairing of the "guide" strand between bases 2-5 on the target RNA, following which cleavage occurs (Ma, Yuan et al. 2005, Frank, Sonenberg et al. 2010) via $Mg^{2+}$ catalyzed hydrolysis, generating the characteristic 3'-hydroxyl and 5'-phosphate termini (Martinez and Tuschl 2004, Schwarz, Tomari et al. 2004). This hinge-like structure allows the protein to accept different-sized RNA molecules as guide strands, allowing flexibility and participation in guide amplification (Wang, Juranek et al. 2008).

### Catalytically active Piwi proteins

Not all Piwi proteins are catalytically active, for example, Piwi in *D. melanogaster* was initially thought to have slicer activity (Saito, Nishida et al. 2006) just as the Piwi protein in *Bombyx mori* (Siwi) does (Matsumoto, Nishimasu et al. 2016), however more recent studies of the purified protein showed that instead of the canonical DEDX motif, Piwi in *D.*

*melanogaster* has a DVDK motif (Darricarrère, Liu et al. 2013, Yamaguchi, Oe et al. 2020). This amino acid change has destroyed the slicer activity of the protein, potentially specializing for its role of piRNA-guided co-transcriptional silencing, as a mutant DEDH Piwi protein dissociates from its target in experimental settings (Yamaguchi, Oe et al. 2020).

Aub and Ago3, the remaining PIWI proteins in *D. melanogaster* have been shown to have slicer activity *in vitro*. (Gunawardane, Saito et al. 2007). While this has not been confirmed using X-ray crystallography to determine if the protein structure matches a known cleavage-competent structure, the endonuclease activity would make sense for the amplification of the piRNA pool through the ping-pong amplification cycle. Both Ago3 and Aub were shown to cleave the target RNA between nucleotides 10 and 11, generating Ago3 piRNAs with an A10 signature and Aub piRNAs with a U1 signature and significant overlap in the first 10 bases (Gunawardane, Saito et al. 2007). In humans, only Argonaute 2 (hAgo2) has confirmed catalytic activity, while hAgo1, hAgo3 and hAgo4 do not (Meister, Landthaler et al. 2004, Yuan, Pei et al. 2005).

## Diversity of PIWI proteins in the animal kingdom

Argonaute proteins are found in almost all eukaryotes, and exhibit relatively high homology across taxa, indicating that these proteins function in conserved pathways, possibly in the last common ancestor of eukaryotes (reviewed in (Hutvagner and Simard 2008, Swarts, Makarova et al. 2014). Prokaryotic Argonaute (pAgo) proteins are structurally similar to

eukaryotic Argonaute proteins, though some are truncated, are found in various bacteria and archaea species with the long pAgos and are involved in host defense against invading RNA (reviewed in (Koopal, Mutte et al. 2023).

A survey of dipteran transcriptomic data shows that there are multiple duplications of Ago2 and Piwi/Aub proteins in various insect species, however, there is only one copy of Ago1 in all Dipterans and Ago3 is only rarely duplicated (Lewis, Salmela et al. 2016). Additionally, somatic expression of piRNAs and the associated Piwi proteins was determined not to be novel, with the current theory being that somatic piRNAs were present in the last common ancestor of all arthropods, with independent lineage-specific losses over time (Lewis, Quarles et al. 2018). This evolutionary context shows that *D. melanogaster* is a good model for other Drosophilidae, however, dipterans in the sub-group Nematocera show marked expansion of Piwi/Aub homologous proteins (Lewis, Salmela et al. 2016) with the aphid (*Acyrthosiphon pisum*) particularly remarkable in that it expresses two paralogs of Ago3 and eight of Piwi with differential expression under different reproductive strategies in the aphid (Lu, Tanguy et al. 2011, Lewis, Salmela et al. 2016).

Rapid gene turnover and protein level diversification in Ago2 and Piwi/Aub may point to potential diversification in function in these different dipterans, a result seen in *D. melanogaster* Ago2, where duplication resulted in functional and tissue specialization of the pattern of expression of the newer Ago2 paralogs, with expression restricted to the testes

(Lewis, Webster et al. 2016). This is intriguing given that the Piwi sub-family experienced an expansion in ancestral Culicine mosquitoes but not in Anopheline mosquitoes, with six Piwi proteins in *C. pipiens* and seven Piwi proteins annotated in the *Ae. aegypti* genome (Campbell, Black et al. 2008). The spatial and temporal distribution of PIWI proteins in *Ae. aegypti* development was established using next-generation RNA sequencing. (See Fig. 2.1) (Akbari, Antoshechkin et al. 2013)

## PIWI function

The main function of an Argonaute protein is the regulation of gene expression which occurs at two levels based on the cellular location. In the cytoplasm these proteins function to silence gene expression by degradation of the transcript or inhibition of translation apparatus assembly, while in the nucleus, gene expression is moderated by the deposition of repressive marks on histones or on the DNA itself in the form of DNA methylation. (Sienski, Dönertas et al. 2012, Le Thomas, Rogers et al. 2013, Le Thomas, Stuwe et al. 2014)

Post-transcriptional repression: The "guide" RNA species which forms part of the RNA-induced silencing complex (RISC), targets the slicer protein complex to the cognate RNA via complementary base-pairing where the target RNA is cleaved between the 10$^{th}$ and 11$^{th}$ base from the 5' end of the "guide" RNA. Experimental studies with purified Ago2 and a synthetic siRNA showed that the Argonaute protein is sufficient to carry out the targeting and cleavage of the target transcript (Rivas, Tolia et al. 2005).

In the *D. melanogaster* ovary and testes, Aub and Ago3 are expressed in the germline cell cytoplasm where the two proteins interact to cleave target RNA molecules and amplify the response via a ping-pong amplification cycle, while Piwi is expressed in the nucleus of both the germline and surrounding somatic cells where silencing of target occurs via histone modification (discussed in greater detail in Chapter 3).

In *Ae. aegypti*, experiments in Aag2 cell culture implicate Piwi5 and Ago3 as the proteins responsible for the production of alphavirus vpiRNAs (Miesen, Girardi et al. 2015). Piwi6 has also been shown to be involved in addition to the other two in flaviviral vpiRNA production (Miesen, Ivens et al. 2016). Piwi4 has been alternately suggested to associate with the siRNA pathway via Ago2 and Dicer-2 proteins and the piRNA pathway, shown by the enhanced viral replication after knockdown experiments in Aag2 cell culture, as well as depleted SINV-piRNAs in Piwi4 knock-down experiments. (Schnettler, Donald et al. 2013, Varjak, Donald et al. 2017).

Aag2 cells are a popular tool to study mosquito arboviral transmission and were made sometime in the 1960s by homogenizing embryos, with later characterization suggesting the cells represent a variety of embryonic tissues (Lan and Fallon 1990). The advent of newer technologies such as cell sorting have resulted in the creation of a clonal population of Aag2 cells, known as Aag2-AF5 which has helped standardize cell culture work in the yellow fever mosquito (Fredericks, Russell et al. 2019). Cell culture, however, does not recapitulate the

molecular environment of the various tissues involved in arbovirus transmission, as illustrated in Qu et al where piRNA expression from defined clusters in Aag2 cells was compared to expression of the same clusters in Ae. aegypti tissues such as the midgut (Qu, Betting et al. 2023).

## CHAPTER AIMS

The expansion of the piRNA pathway associated PIWI protein repertoire in *Ae. aegypti* points to possible divergence and functional specialization in the yellow fever mosquito. The goal of this project was to interrogate the potential redundancy present in the *piwi* genes functions in whole insects, unlike previous studies which only interrogated cell culture. This was done by first attempting to create knockout lines of each of the target *ago3* and *piwi 2, 4-6* genes, chosen due to their expression outside of embryonic tissue, using CRISPR-Cas9 gene editing technology. Observation of key characteristics such as hatching and survival of the potentially mosaic G0 mosquitoes was used to infer the lethality of each of the target genes, coupled with testing for inheritance of mutant alleles.

An additional goal of this project was the identification of the binding partner for Ago3 in larval somatic tissues. This was carried out by dissections of the midgut, which is an important tissue involved in viral transmission, as it represents a barrier to entry into systemic circulation for viruses acquired via ingestion , followed by immunoblotting for the

presence of Piwi2-7, to confirm earlier transcript-level evidence for stage-specific expression of these proteins (Akbari, Antoshechkin et al. 2013, Han 2017).

This will be the first such study carried out on the juvenile stage of *Ae. aegypti* mosquitoes. Larvae are often overlooked as the blood-feeding adult female stage draws the most attention, due to the nuisance associated with the bite and the arboviruses transmitted by some mosquitoes. This represents a missed opportunity to interrogate the mosquito-virus interactions that occur because of the voracious feeding the larvae partake in during the aquatic stage of their growth and development. Understanding the molecular details of this relationship provides the field of mosquito vector control with a better understanding of how to exploit mosquito biology against arboviral transmission.

## MATERIALS AND METHODS

### Strain - Exu-Cas9

The mosquitoes used for all experiments in this chapter express Cas9 under the control of a germline promoter, *Exuperentia* (Exu) known as Exu-Cas9. (Li, Bui et al. 2017). The expression of this gene is triggered by blood-feeding, after which it is expressed in the ovary and early embryo. This strain was chosen due to the increased editing efficiency and reduced injection load, as Cas9 does not need to be included, which should result in greater survival of the injected animal.

## Rearing protocol – general

*Ae. aegypti* mosquitoes were reared in an ACL2 insectary maintained at approximately 26-28°C and 60-80% humidity. Mosquito eggs were hatched by immersing dried filter paper with eggs attached in deionized water (DI water). A pinch of mosquito food – Tetramin tablets (Tetra, Spectrum Brands Pet, LLC, Blacksburg, VA) ground into powder was added to the hatching cup which is then placed in a vacuum chamber. The eggs were hatched under vacuum for a minimum of 2 hours after which the hatching cup was observed for evidence of LI larvae. The egg cup was then removed from the vacuum chamber and the hatched larvae were placed into a larval tray. Approximately 200 larvae were placed per larval pan with 1 L of DI water and a pinch of crushed Tetramin tablet. Larvae were fed every other day and monitored for the presence of excess food in the tray to prevent overgrowth of bacteria and fungi which interferes with the growth of the larvae. The amount of food given to the tray was increased as the larvae moved through L3 and L4 larval stages of growth associated with voracious feeding.

After approximately five days, the larvae begin to pupate, with adults emerging 48 hours after pupation. Pupae were picked using a 2 mL plastic pipette and transferred to the adult cages (BugDorm-1; MegaView Science Co., Ltd., Taichung, Taiwan) in small plastic 30 mL medicine cups. One large cage should hold around 400 mosquitoes i.e., two larval trays.

The adult mosquitoes were provided with carbohydrate source *ad libitum* (10% sucrose in DI water placed in a lidded plastic jello cup with a dental cotton plug to act as a wick). The adults were given 2-4 days to mate before being provided with a blood meal using an artificial blood feeding system composed of glass bulbs through which water was pumped through using the Thermo Scientific SC100-S7 4-7 L Heated Bath (Cole-Parmer, Vernon Hills, Illinois). Defibrinated bovine or sheep's blood held in place by Parafilm® M was warmed to 37°C and the adults were allowed to feed for at least 4 hours. If the cage was being fed for injections, it was fed on two consecutive days to increase the chances that females will feed till satiety.

72 hours after blood-feeding the mosquitoes were provided with an egg cup – plastic soup cup with approximately 20 mL of water lined with wet filter paper. The adults were given up to four days to lay eggs after which the egg cup was removed from the cage. All water was removed and the filter paper with eggs was allowed to dry. Once completely dry, the eggs were stored by carefully wrapping the filter paper with tissue paper and placing it in a sealed plastic bag. The eggs remain viable for 6 months and sometimes up to a year if stored in a humid environment.

The mosquito colony was screened in the pupal stage for the presence of the Exu-Cas9 cassette as marked by OpIE2-dsRed until the majority had the RFP+ that meant that they contained the plasmid with Exu-Cas9. Once this mostly homozygous line was established,

screening was done every few generations to maintain the high level of expression, as judged by brightness of the dsRed.

### sgRNA design and construction

Due to the relatively few bioinformatic tools designed specifically for *Ae. aegypti* several redundant methods were used to design sgRNAs. The sequence of the first coding exon of each *piwi* gene was chosen as the target where possible. ChopChop bio (https://chopchop.cbu.uib.no/) was used to design several sgRNAs per target, CRISPRScan (https://www.crisprscan.org/) was used to identify the potential off-targets in greater detail than ChopChop bio could. sgRNA candidates were then checked for uniqueness using BLASTn against the AaegL4/5 genome build in VectorBase (https://vectorbase.org/vectorbase/app).

Two sgRNAs were chosen per target *piwi* genes to increase the probability of long deletions between the two cut sites (Li, Bui et al. 2017). This also enabled the identification of large deletions in mosaic G0 mosquitoes as there would be two amplicons in the PCR of the region surrounding the sgRNA target sites – one for the WT sequence and one for the deletion allele.

For the first two sets of injections, the sgRNAs were synthesized in lab as follows:

The sgRNA template primer and the Universal primer ordered from Integrated DNA Technologies, Inc. (IDT; Coralville, IA) were used to make the sgRNA DNA template. This was purified and prepped for *in vitro* transcription using the MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA) following the manufacturer's instructions. The RNA product was treated to remove any residual DNA using Turbo DNase (Thermo Fisher Scientific Inc., Waltham, MA), and quality tested on a Bioanalyzer (UCR IIGB Genomics Core) to ensure the only RNA in the sample would be the sgRNA.

The CRISPaint experiment II injections used sgRNAs ordered from Synthego (Redwood City, CA) with the standard suite of modified bases.

Several sgRNAs were chosen per target and each was investigated for off-target efficiency (Table 2.1).

### *In vitro* testing of sgRNAs

For each guide, *in vitro* testing was performed to determine cutting efficiency using the Guide-it sgRNA Screening Kit (Takara Bio Inc., Shiga, Japan). Following the recommended protocol, the sgRNA cutting efficiency was qualitatively determined by incubating each

sgRNA with the target region amplicon of Exu-Cas9 gDNA and comparing it to a control sgRNA and control DNA fragment.

### Injection protocol

Microinjection following (Kistler, Vosshall et al. 2015, Li, Bui et al. 2017) with modifications from (Li, Bui et al. 2017)

Filamented quartz needles with the following dimensions: outside diameter 1.00 mm, inside diameter 0.7 mm, length of 10 cm. (Sutter Instrument Company, Novato, CA) were siliconized by coating with Sigmacote® (Sigma-Aldrich, Inc, St. Louis, MO) then baked overnight in an oven to get rid of excess reagent. The needles were pulled using the P-2000 (Sutter Instrument Company, Novato, CA) laser puller with the following settings: heat: 800 filament 4, pull 160, velocity 45, delay 135. The point of the needles was opened using the BV-10 micropipette beveler (Sutter Instrument Company, Novato, CA) with the 104 very fine stone at an angle of 30°.

Whatman ® qualitative filter paper, Grade 1 (Sigma-Aldrich, Inc, St. Louis, MO) was used to line up eggs after wetting with DI water, after which the embryos were transferred to microscope cover slips 22 mm x 22 mm, (Bel-Art - SP Scienceware, Wayne, NJ) with double-sided tape and covered with halocarbon oil 27 (Sigma-Aldrich, Inc, St. Louis, MO). 2 μL of injection mix was backloaded into needles using the Eppendorf micro-loader tips (VWR, Radnor, PA). Injections were performed on the XenoWorks™ Digital Microinjector (Sutter

Instrument Company, Novato, CA), range set to 2, with the needle held at around 15° maximum angle. Inject and transfer pressures were varied depending on internal pressure in the embryo being injected,

### Rearing injected mosquitoes

After blood-feeding, there were 2-3 days of injections per blood-fed cage. 3 days after feeding, approximately 30 gravid females were collected using a mouth-operated pipette with a filter. The females were transferred to an egg laying apparatus (a 50 mL falcon tube with damp filter paper in it) which was placed in a warm completely dark area and checked for eggs every 30 minutes. Spent females were placed in a different cage.

After 3-4 days, a hatching cup was prepared and the slide with the injected eggs was placed into an egg cup with DI water and a pinch of ground up Tetramin tablet. The egg cup was monitored for hatching with larvae counted as they emerge. Any mosquitoes that died during development were collected as soon as found and frozen for DNA later extraction.

Screening for any fluorescence as was necessary in PIWI CRISPaint experiments 2 and 3, was done at the pupal stage. Pupae were collected and screened by looking for fluorescence in the developing eye. Pupae were chosen for screening as they are easier to handle than larvae or adults, and the eye can be easily visualized with minimal manipulation of the mosquito.

Fluorescent screening was done using a Leica M165 FC Fluorescent Stereo Microscope (Leica Microsystems Inc., Deerfield IL), visualization was performed in ToupView software (http://www.touptek.com/product/showproduct) and pictures were taken using the XCAM4K UHD camera (Zhejiang, China).

### Molecular analysis of G0s

Genomic DNA (gDNA) was extracted from single mosquitoes using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Venlo, The Netherlands) and following the insect protocol with one modification: the incubation at 56°C step was done overnight for approximately 16 hours to allow for full digestion of the cuticle. The DNA was quantified using the Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) and used as a template in PCR.

Primers were constructed either by hand or using Primer3 (Koressaar 2007, 2012) where possible. Primer sequences were tested for off-target matches using BLASTn algorithm in VectorBase to confirm the specificity of the primes to the target site. The unique primers were then ordered from IDT with standard purification (Integrated DNA Technologies, Inc, Coralville, IA) (Table 2.2). Primer cycling conditions were determined using the NEB Tm calculator (https://tmcalculator.neb.com/) and confirmed via a test PCR on the background strain, Exu-Cas9 gDNA, before amplifying the target region of the experimental samples. A high-fidelity polymerase, Q5, was used for genomic DNA amplification with

dNTPs supplied by NEB. (New England Biolabs, Ipswich, MA). The details of the PCR amplicon sizes can be found in Table 2.3.

The T7 endonuclease I (T7E1) assay (New England Biolabs, Ipswich, MA) was used to narrow down the pool of G0 mosquitoes sent for sequencing in the NHEJ and CRISPaint I experiments. PCR amplicons are cleaned of extra primers etc using Qiagen PCR purification kit (Qiagen, Venlo, The Netherlands). The T7E1 assay results in the cleaving of mismatched double-stranded DNA, such as would occur from the repair of a double-stranded break caused by Cas9.

Any positive G0 mosquitoes identified using the T7E1 were prepared for sequencing by first cloning PCR amplicons into the pJet1.2/blunt vector using the CloneJET PCR Cloning kit (Thermo Fisher Scientific Inc., Waltham, MA) which was then transformed into *E. coli* following the manufacturer's instructions. The bacteria were allowed to grow for a maximum of 16 hours overnight, after which single colonies were picked. Colony PCR was used to amplify out the insert which was then purified using the Qiagen PCR purification kit and sent out for Sanger sequencing.

To characterize any indels present in the experimental mosquitoes, the sequence was checked against the reference Liverpool genome in VectorBase using BLASTn (https://vectorbase.org/vectorbase/app).

## Midgut pattern of PIWI protein expression

Approximately 30 L4 larvae were dissected in cold PBS and the midguts placed in protein extraction solution consisting of Readyprep sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) with Halt Protease Inhibitor Cocktail (Thermo) and Phosphatase Inhibitor Cocktail 3 (sigma), both at 3x concentration unless otherwise indicated. Additional tissues, adult testes, and adult ovaries were dissected to serve as controls as the pattern of Piwi expression is known in the adult reproductive tissues, and whole L4 larvae to compare expression in the larval midgut with the whole animal at this specific developmental stage.

The samples were mechanically disrupted using a motorized pestle holder (Kimbel Chase Life Science and Research Products, Vineland, NJ) and disposable sterile plastic pestles, then spun in a refrigerated table-top centrifuge at top speed (approximately 20,000g). The supernatant, which contained the protein fraction was transferred to another microfuge tube and the protein sample was quantified using the Qubit Fluorometer 2.0 with the Qubit protein assay kit following the manufacturer's instructions (Thermo Fisher Scientific Inc., Waltham, MA).

The protein samples were mixed with the 2X Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) diluted according to manufacturer's instructions with added b-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri) to serve as a reducing agent, then

boiled at 98C for 8 minutes in a thermocycler with a heated lid. The samples were allowed to cool to room temperature before loading onto the gel. Polyacrylamide gel electrophoresis (PAGE) was carried out using 4-15% Mini-PROTEAN® TGX™ Precast Gels with tris-glycine-SDS running buffer and Precision Plus Protein Dual Color standard serving as the molecular weight marker (Bio-Rad Laboratories, Inc., Hercules, CA) at 90V until the dye front reached the bottom of the gel.

The gel was removed from the cassette and equilibrated in Tris-glycine-methanol for around 15 minutes, while the PVDF membrane was activated in 100% methanol for 3 minutes. (Bio-Rad Laboratories, Inc., Hercules, CA). The transfer sandwich was set up to transfer the proteins to the PVDF membrane overnight at 4°C at a constant 80 Amps. Successful transfer was confirmed by the presence of the pre-stained protein standard on the membrane. The membranes were rinsed in tris-buffered saline with Tween-20 (TBST) on the rocker 3 times for 5 minutes each. The membrane was then blocked in 5% non-fat dry milk in TBST on the rocker for 1 hour at room temperature, or overnight at 4C. After blocking, the membrane was incubated in the primary PIWI antibody diluted in 5% non-fat dry milk in TBST at the stated concentration. The primary incubation was done for 1 hour at room temperature or overnight in the cold room. The membrane was then washed 3 times in TBST for around 10 minutes each. The loading control used was α-tubulin. The secondary antibody goat anti-rabbit HRP (Bio-Rad Laboratories, Inc., Hercules, CA), was diluted to 1:10,000 in 5% non-

fat dry milk in TBST and incubated similarly to the primary antibody. The membrane was rinsed as before in TBST and prepared for chemiluminescent imaging using Pierce ECL Western Blotting Substrate and the blots were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

## RESULTS

### AGO3

*in vitro* sequencing results showed that all three sgRNAs were effective (Fig. 2.2). The sgRNAs chosen for *in vivo* editing were Ago3 sgRNAs 1 and 3. The NHEJ experiment results are illustrated in Table 2.4, where the hatch frequency was 11.5%. Screening PCR amplicons by T7E1 and sequencing of clones resulted in two mutant alleles, both at sgRNA3, a nine bp deletion in a larva that died in the L3 developmental stage, and a sixteen bp deletion in an adult male that died soon after eclosion (Fig. 2.3A). In the CRISPaint experiment I (Table 2.5), the hatch frequency was 4.8%, while in the second CRISPaint experiment, the hatch frequency was a lot higher at 25.6% (Table 2.6), prompting a third set of Ago3 CRISPaint injections to resolve the disparity. The third set of Ago3 CRISPaint injections are illustrated in Table 2.7, where the hatch frequency was 22.8%.

PIWI2

While *in vitro* sequencing results (Fig. 2.2) showed that only sgRNA2 was effective at cleaving the target amplicon, mutations were recovered *in vivo* at sgRNA1 as shown in Fig. 2.4B. All Piwi2 injections had similar hatch frequencies of between 12% – 17% (Tables 2.4, 2.5, 2.6). Only the CRISPaint II experiments resulted in evidence of editing with one mosquito positive for fluorescence (Fig. 2.4E) giving a mutagenesis rate of 2.6% of hatched G0 larvae. ICE analysis of Sanger sequencing of the PCR amplicon revealed three different 510 bp deletion alleles, reflecting deletion of the region between the two sgRNAs (Fig. 2.4C).

PIWI4

Both Piwi4 sgRNAs showed efficient cleavage with the *in-vitro* cleavage assay. For both the NHEJ and CRISPaint I experiments, some individual mosquitoes showed up as positive on the T7 Endonuclease I assay but did not show up in Sanger sequencing of clones, possibly pointing to a very low frequency of mutation in the mosaic G0. Indels were confirmed at both sgRNAs (Fig. 2.5). Piwi4 injected embryos exhibited low hatch frequencies across all three injection experiments.

PIWI5

As with Piwi4, both Piwi5 sgRNAs exhibited efficient cleavage of the target amplicon. Five T7E1 mosquitoes were recovered, and one male was confirmed *via* Sanger sequencing. Interestingly this male mosquito had a variety of alleles including a deletion between the two sgRNAs, which are approximately 500bp apart (Fig. 2.6A).

The CRISPaint I experiment resulted in two GFP+ mosquitoes (Fig. 2.6C), which eventually eclosed to give a male (left panel) and one female (right panel) mosquitoes. These were back-crossed to the Exu-Cas9 strain and G1 was screened for presence of the GFP fluorescence in the eye, with none observed. The CRISPaint II experiment was also successful in Piwi5 with three individual G0 mosquitoes testing positive for cleavage by PCR screening (Fig. 2.6C).

PIWI6

A variety of alleles were recovered from the positive G0 mosquitoes in the NHEJ experiment, including a 187 bp deletion which eliminated one sgRNA site and a 30 bp deletion that abolished the other sgRNA target site (Fig. 2.7A). This gives an editing efficiency of 15.6% of hatched larvae.

In the first CRISPaint experiment, two pupae positive for RFP fluorescence were obtained during screening (Fig. 2.7B). insertion of the plasmid at the target site was confirmed for the pupa on the left by junction PCR across the plasmid-gene junction (Fig. 2.7C), giving a

mutagenesis rate of 4.5% of hatched larvae. the RFP+ phenotype proved to be difficult to screen for in dsRed positive mosquitoes, therefore, the CRISPaint-GFP plasmid was used exclusively going forward. The CRISPaint II experiment did not yield any G0 mosquitoes that were PCR positive.

### WHITE

While no G0 GFP+ mosquitoes were recovered, screening of the G1 pupae resulted in one GFP+ pupa (Fig. 2.8). Interestingly, the G1 pupa still retained most of the eye pigment. This is the first example of inheritance in the CRISPaint experiments.

### Midgut-specific protein experiments

Blotting was performed for each Piwi protein in larval midgut with the adult ovaries and testes serving as positive controls. Interestingly, Piwi4 is smaller than expected, approximately 20 kDa, when the calculated size is 95 kDa. Increasing the protease and phosphatase inhibitor concentrations up to 7x did not lead to recovery of the full-sized protein, indicating perhaps the cleavage was not due to enzymatic digestion (Fig. 2.9). A comparison of Piwi4 size across the tissues tested is illustrated in Fig. 2.10 where the adult tissues have full length protein while the juvenile tissues do not. Further analysis of the gel slice between 15 kDa and 25 kDa by mass spectrometry identified 25 peptides from Piwi4, confirming that the antibody is specific to Piwi4, and the smaller band is not due to off-target binding activity of the antibody.

The calculated size of Piwi5 is 97 kDa. In Fig. 2.11, Piwi5 appears to be absent from whole larvae but is present in the midgut protein sample. This potentially indicates that Piwi5 is only expressed in the midgut in L4 larvae (pending MS of the band). Piwi6, which is calculated to be 97.7 kDa, was present only in the ovaries (Fig. 2.12). Piwi2 is present in blood-fed ovaries as expected, and was also present in testes, which was not expected from previous RNA-seq data (Fig. 2.13). Ago3 was only detected in the blood-fed ovaries (Fig. 2.14), although transcripts were detected in the male as well as the larval stages of development.

## DISCUSSION

In the search for new tools for use in the control of vector-borne diseases, *D. melanogaster* is typically used as the model for elucidating RNAi pathways in all insect vectors, however in this chapter I demonstrate that when it comes to the piRNA pathway and its potential role in antiviral immunity, the fruit fly is not an appropriate choice of model organism.

The Piwi gene family in *Ae. aegypti* has undergone an evolutionary expansion relative to the same in *D. melanogaster*. This has led some to believe that Piwi in *Ae. aegypti* is redundant. The fruit fly expresses three Piwi sub-family proteins - Piwi, expressed in the nucleus of germline cells, Ago3 and Aubergine expressed in the cytoplasm of the supporting follicular cells (Brennecke, Aravin et al. 2007).

Analysis of the evolutionary history of Piwi sub-family genes in Dipterans revealed a duplication in Piwi leading to functional specialization into Piwi and Aub in the *D. melanogaster* lineage that did not occur in Nematocera sub-order of Dipterans, which includes mosquitoes (Lewis, Salmela et al. 2016). *Ae. aegypti* encodes 8 Piwi sub-family genes: Piwi1-7 and Ago3, with expression in both germline and somatic tissues, as confirmed by small RNA-seq (see Fig. 2.1). Developmental stage specific RNA-seq analysis of Piwi expression in *Ae. aegypti* revealed that Piwi 4-6 and Ago3 are expressed in both germline and somatic tissues, while Piwi 1-3 are expressed in the germline, and Piwi7 is mostly expressed in the developing embryo (Akbari, Antoshechkin et al. 2013). As most investigations into the functions of the Piwi sub-family of proteins have been carried out in cell culture, information on the tissue and developmental-stage specific expression of Piwi proteins is lacking. I aim to determine the pattern of Piwi protein expression in larvae with a focus on the larval midgut.

The larval stage of development is of special interest due to the midgut epithelium representing the first barrier faced by viruses ingested during one of the two life stages associated with voracious feeding and exposure to viruses via ingestion; the other being the adult female that is exposed to blood-borne pathogens. The adult male feeds on nectar, which is stored in the crop, not the midgut (Clemments 2000). The larval stage is under investigated

in favor of the adult female which takes a bloodmeal from vertebrate hosts and vectors diseases, therefore the former was chosen as the developmental time frame for study.

To investigate potential redundancy in the Piwi sub-family, CRISPR-Cas9 was used to generate knockout lines. Injections targeting each piwi gene were performed to create transgenic lines with deletions in each somatically expressed Piwi, that is, *Piwi4-6* and *Ago3*, as well as one germline Piwi (*Piwi2*), and a control gene (*white* or *ebony*) to determine if the genes are functionally redundant. This is because *D. melanogaster* encodes for one Piwi protein, leading to speculation that the PIWI family expansion seen in *Ae. aegypti* is due to gene duplications resulting in paralogs with similar function. Contrary to this belief, I demonstrate evidence of functional specialization, as inferred by the differences in hatching and survival of hatched larvae frequencies observed.

A limitation of this study was that no lines were made, therefore the G0 transgenic mosquitoes were mosaic for the deletions with a variety of alleles present in one mosquito. The hatch, eclosion and survival frequencies are illustrated in Fig. 2.15.

This non-redundancy points to specialized functions of these genes perhaps in a time and tissue-specific manner, providing *in vivo* confirmation for the functional specialization of Piwi proteins observed during transient knock-down experiments in Aag2 cell culture (Miesen, Girardi et al. 2015). In the results from my knockout experiments in the mosquito, Piwi4 has consistently statistically significantly low hatching frequencies when compared to

the control in each experiment, whereas Ago3 and Piwi2 have statistically significantly low hatching frequencies compared to White in the NHEJ experiment, and Piwi5 hatching frequency is also significantly low compared to the control in the CRISPaint II experiment (Fig. 2.16).

The low hatching observed in Piwi2 knockout injections is consistent with the RNA-seq data (Akbari, Antoshechkin et al. 2013) indicating that it is expressed in blood-fed ovaries. Immunoblotting revealed that surprisingly, Piwi2 was detected in both testes and 72H-post blood-feeding ovaries (Fig. 2.13). Perhaps Piwi2 is only expressed in the testes which is why RNA-seq of the whole male did not detect the Piwi2 transcript. Altogether this indicates that Piwi2 is potentially necessary for the developing embryo, and therefore Piwi2 mutants are embryonically lethal.

Other knockout experiments in Aag2 cell culture indicate that Piwi5 and Ago3 participate in antiviral piRNA biosynthesis in the cytoplasm, while Ago3, Piwi5, Piwi6 and to a lesser extent Piwi4, participate in transposon-specific piRNAs (Miesen, Girardi et al. 2015). Piwi4 was found to be depleted of both viral and transposon piRNAs in one study (Miesen, Girardi et al. 2015), but knockdown of Piwi4 in a different study led to an increase in viral replication though there was no significant effect on viral piRNA expression (Varjak, Maringer et al. 2017). Additional studies in cell culture showed that EVE-derived piRNAs associated with

Piwi4 and this Piwi4-piRISC was efficient at silencing the replication of the cognate virus during acute infection (Tassetto, Kunitomi et al. 2019).

In the Piwi protein expression pattern experiments, truncated versions of Piwi4 and Piwi5 were detected in the larval stage, while Piwi6 was not detected. My data calls into question whether Piwi6 is necessary for silencing the activity of transposable elements, as Piwi6 was not detected in L4 larvae.

Additionally, Ago3 protein was only detected in blood-fed ovaries, and surprisingly not in adult testes, raising the possibility that ping-pong amplification of piRNAs is limited to the female germline and the developing embryo. Ago3 is a key partner of the ping-pong amplification pathway, where the piRNAs with a 10A bias derived from the sense strand of the piRNA cluster and bound to Ago3 are used as a template for Aub-bound piRNAs in *D. melanogaster*. The presence of the ping-pong pathway in *Ae. aegypti* ovaries but not the testes, as inferred from lack of Ago3 protein in the testes, makes sense from a biological perspective as many entomopathogenic viruses are trans-ovarially transmitted. This vertical transmission is documented feature of how some ISVs spread throughout a mosquito population (Lutomiah, Mwandawiro et al. 2007, Saiyasombat, Bolling et al. 2011)(Lutomiah 2007, Rungrat Saiyasombat 2011). Signatures of ping-pong biogenesis have been observed in cell culture and in *Ae. aegypti* ovaries, however, the absence of Ago3 from larval tissues

supports recent observations that somatic piRNAs are predominantly primary piRNAs with the characteristic 1U bias (Qu, Betting et al. 2023).

Interestingly, the Piwi4 protein is truncated in *Ae. aegypti* larval tissues as confirmed by mass spectrometry. The epitope recognized by the Piwi4-antibody is located at the N-terminal of the protein, therefore the approximately 20 kDa Piwi4 fragment recovered in larvae would indicate that the N-terminal of the protein has been cleaved. The N-terminal of Piwi4 is known to contain a nuclear localization signal, and the RNA-binding domain and the catalytic cleavage domains are located towards the C-terminal of the protein (Williams, Shrivastava et al. 2021). Cleavage of the N-terminal domain could confine Piwi4 to the cytoplasm where the rest of the protein is potentially active and participating in the production of viral piRNAs in the larval midgut. Alternatively, the cleavage could be the result of enzymatic activity that was not restricted by up to 7x protease inhibitors used during protein extractions.

*D. melanogaster* is typically used as a model for many other systems in insects. This work demonstrates that the fruit fly is not a good model for the piRNA pathway in insects, and advocates for caution in choice of model organism for elucidation of the mechanisms of specific biological systems in insects. Piwi sub-family gene expression in *D. melanogaster* is confined to the germline and supporting somatic cells with the functions of these genes seemingly limited to the protection of the germline from mobile genetic elements.

# CONCLUSION

RNAi is an important function of life for regulation of self and protection of the germline from foreign genetic elements. An RNA-guided RNA targeting complex (RISC) is crucial for life as evidenced by the conservation of the proteins involved in the RNAi pathway and the expansion of these proteins in certain taxa followed by co-option for their own needs. The role of PIWI extends beyond germline protection in the mosquito *Aedes aegypti*. The multiple PIWI genes are not mere duplicates performing the same function as shown by the variable outcomes of the KO experiments, as well as the absence of some of the proteins from specific developmental stages.

The only Piwi sub-family proteins detected in the larval stage of development are a truncated form of Piwi4 and Piwi5. This indicates that the ping-pong biogenesis pathway is not active in larvae, as Ago3 is a key partner in the pathway, supporting previous observations that the piRNA pathway in somatic tissues is mostly carried out *via* the primary biogenesis pathway, as evidenced by the 1U bias of Piwi-bound piRNAs. Full length Ago3, Piwi2, Piwi4, Piwi5 and Piwi6 proteins were detected in blood-fed ovaries, indicating that the ping-pong biogenesis pathway may occur in the female germline cells. The situation in the testes is slightly unexpected with Ago3 not detected, perhaps indicating that ping-pong biogenesis does not occur in the male gonads.

While a piwi knockout line was not established, observation of the G0 mosquitoes during development provides some support for the conclusion that the *piwi* genes tested are necessary for development. More work is needed to untangle the individual and potential concerted functions of Piwi proteins in the yellow fever mosquito with attention to the different tissues and developmental stages.

The characterization of Piwi proteins expression during the larval stage provides the vector control field with potential targets which could be co-opted to equip the mosquitoes with the ability to suppress replication of arboviruses of medical importance, as these proteins have been confirmed to be antiviral in the mosquito ovaries. This would in turn limit the transmission of these viruses, potentially resulting in a reduction in the arboviral disease burden.

BIBLIOGRAPHY

Abraham, E. G., M. Donnelly-Doman, H. Fujioka, A. Ghosh, L. Moreira and M. Jacobs-Lorena (2005). "Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements." Insect Mol. Biol. **14**(3): 271-279.

Adams, K. L., D. G. Abernathy, B. C. Willett, E. K. Selland, M. A. Itoe and F. Catteruccia (2021). "Wolbachia cifB induces cytoplasmic incompatibility in the malaria mosquito vector." Nat Microbiol **6**(12): 1575-1582.

Adelman, Z. N., I. Sanchez-Vargas, E. A. Travanty, J. O. Carlson, B. J. Beaty, C. D. Blair and K. E. Olson (2002). "RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome." J. Virol. **76**(24): 12925-12933.

Aguiar, E. R. G. R., J. P. P. de Almeida, L. R. Queiroz, L. S. Oliveira, R. P. Olmo, I. J. d. S. de Faria, J.-L. Imler, A. Gruber, B. J. Matthews and J. T. Marques (2020). "A single unidirectional piRNA cluster similar to the flamenco locus is the major source of EVE-derived transcription and small RNAs in Aedes aegypti mosquitoes." RNA **26**(5): 581-594.

Akbari, O. S., I. Antoshechkin, H. Amrhein, B. Williams, R. Diloreto, J. Sandler and B. A. Hay (2013). "The developmental transcriptome of the mosquito Aedes aegypti, an invasive species and major arbovirus vector." G3 **3**(9): 1493-1509.

Amos, B., C. Aurrecoechea, M. Barba, A. Barreto, E. Y. Basenko, W. Bażant, R. Belnap, A. S. Blevins, U. Böhme, J. Brestelli, B. P. Brunk, M. Caddick, D. Callan, L. Campbell, M. B. Christensen, G. K. Christophides, K. Crouch, K. Davis, J. DeBarry, R. Doherty, Y. Duan, M. Dunn, D. Falke, S. Fisher, P. Flicek, B. Fox, B. Gajria, G. I. Giraldo-Calderón, O. S. Harb, E. Harper, C. Hertz-Fowler, M. J. Hickman, C. Howington, S. Hu, J. Humphrey, J. Iodice, A. Jones, J. Judkins, S. A. Kelly, J. C. Kissinger, D. K. Kwon, K. Lamoureux, D. Lawson, W. Li, K. Lies, D. Lodha, J. Long, R. M. MacCallum, G. Maslen, M. A. McDowell, J. Nabrzyski, D. S. Roos, S. S. C. Rund, S. W. Schulman, A. Shanmugasundram, V. Sitnik, D. Spruill, D. Starns, C. J. Stoeckert, S. S. Tomko, H. Wang, S. Warrenfeltz, R. Wieck, P. A. Wilkinson, L. Xu and J. Zheng (2022). "VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center." Nucleic Acids Res. **50**(D1): D898-D911.

Andersen, P. R., L. Tirian, M. Vunjak and J. Brennecke (2017). "A heterochromatin-dependent transcription machinery drives piRNA expression." Nature **549**(7670): 54-59.

Ant, T. H., C. S. Herd, V. Geoghegan, A. A. Hoffmann and S. P. Sinkins (2018). "The Wolbachia strain wAu provides highly efficient virus transmission blocking in Aedes aegypti." PLoS Pathog. **14**(1): e1006815.

Ant, T. H. and S. P. Sinkins (2018). "A Wolbachia triple-strain infection generates self-incompatibility in Aedes albopictus and transmission instability in Aedes aegypti." Parasit. Vectors **11**(1): 295.

Aravin, A., D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf, N. Iovino, P. Morris, M. J. Brownstein, S. Kuramochi-Miyagawa, T. Nakano, M. Chien, J. J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan and T. Tuschl (2006). "A novel class of small RNAs bind to MILI protein in mouse testes." Nature **442**(7099): 203-207.

Aravin, A. A., M. Lagos-Quintana, A. Yalcin, M. Zavolan, D. Marks, B. Snyder, T. Gaasterland, J. Meyer and T. Tuschl (2003). "The small RNA profile during Drosophila melanogaster development." Dev. Cell **5**(2): 337-350.

Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky and V. A. Gvozdev (2001). "Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline." Curr. Biol. **11**(13): 1017-1027.

Arensburger, P., R. H. Hice, J. A. Wright, N. L. Craig and P. W. Atkinson (2011). "The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs." BMC Genomics **12**: 606.

Aryan, A., M. A. E. Anderson, K. M. Myles and Z. N. Adelman (2013). "TALEN-based gene disruption in the dengue vector Aedes aegypti." PLoS One **8**(3): e60082.

Asgari, S. (2014). "Role of microRNAs in arbovirus/vector interactions." Viruses **6**(9): 3514-3534.
Atwood, K. C., R. C. Von Borstel and A. R. Whiting (1956). "An Influence of Ploidy on the Time of Expression of Dominant Lethal Mutations in Habrobracon." Genetics **41**(6): 804-813.

Ayettey, J., A. Ablorde, G. K. Amlalo, B. A. Mensah and A. A. Kudom (2023). "Entomological surveillance on Aedes aegypti during covid 19 period in Cape Coast, Ghana: Risk of arboviral outbreaks, multiple insecticide resistance and distribution of F1534C, V410L and V1016I kdr mutations." PLoS Negl. Trop. Dis. **17**(5): e0011397.

Balaji, S., S. Jayachandran and S. R. Prabagaran (2019). "Evidence for the natural occurrence of Wolbachia in Aedes aegypti mosquitoes." FEMS Microbiol. Lett. **366**(6).

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Basu, S., A. Aryan, J. M. Overcash, G. H. Samuel, M. A. E. Anderson, T. J. Dahlem, K. M. Myles and Z. N. Adelman (2015). "Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **112**(13): 4038-4043.

Beckmann, J. F., J. A. Ronau and M. Hochstrasser (2017). "A Wolbachia deubiquitylating enzyme induces cytoplasmic incompatibility." Nat Microbiol **2**: 17007.

Besson, B., O. M. Lezcano, G. J. Overheul, K. Janssen, C. G. Spruijt, M. Vermeulen, J. Qu and R. P. van Rij (2022). "Arbovirus-vector protein interactomics identifies Loquacious as a co-factor for dengue virus replication in Aedes mosquitoes." bioRxiv.

Blair, C. D. and K. E. Olson (2015). "The role of RNA interference (RNAi) in arbovirus-vector interactions." Viruses **7**(2): 820-843.

Blair, C. D., K. E. Olson and M. Bonizzoni (2019). "The Widespread Occurrence and Potential Biological Roles of Endogenous Viral Elements in Insect Genomes." Curr. Issues Mol. Biol. **34**: 13-30.

Bonneau, M., C. Atyame, M. Beji, F. Justy, M. Cohen-Gonsaud, M. Sicard and M. Weill (2018). "Culex pipiens crossing type diversity is governed by an amplified and polymorphic operon of Wolbachia." Nat. Commun. **9**(1): 319.

Bonning, B. C. and M.-C. Saleh (2021). "The Interplay Between Viruses and RNAi Pathways in Insects." Annu. Rev. Entomol. **66**: 61-79.

Bořkovec, A. B. (1976). "Control and Management of Insect Populations by Chemosterilants." Environ. Health Perspect. **14**: 103-107.

Bosch, J. A., R. Colbeth, J. Zirin and N. Perrimon (2019). "Gene knock-ins in Drosophila using homology-independent insertion of universal donor plasmids." bioRxiv.

Bosch, J. A., S. Knight, O. Kanca, J. Zirin, D. Yang-Zhou, Y. Hu, J. Rodiger, G. Amador, H. J. Bellen, N. Perrimon and S. E. Mohr (2020). "Use of the CRISPR-Cas9 System in Drosophila Cultured Cells to Introduce Fluorescent Tags into Endogenous Genes." Curr. Protoc. Mol. Biol. **130**(1): e112.

Bottino-Rojas, V. and A. A. James (2022). "Use of Insect Promoters in Genetic Engineering to Control Mosquito-Borne Diseases." Biomolecules **13**(1).

Brackney, D. E., J. C. Scott, F. Sagawa, J. E. Woodward, N. A. Miller, F. D. Schilkey, J. Mudge, J. Wilusz, K. E. Olson, C. D. Blair and G. D. Ebel (2010). "C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response." PLoS Negl. Trop. Dis. **4**(10): e856.

Breeland, S. G., G. M. Jeffery, C. S. Lofgren and D. E. Weidhaas (1974). "Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. I. Characteristics of the test site and the natural population." Am. J. Trop. Med. Hyg. **23**(2): 274-281.

Brennecke, J., A. A. Aravin, A. Stark, M. Dus, M. Kellis, R. Sachidanandam and G. J. Hannon (2007). "Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila." Cell **128**(6): 1089-1103.

Bushland, R. C. and D. E. Hopkins (1951). "Experiments with Screw-Worm Flies Sterilized by X-Rays." J. Econ. Entomol. **44**(5): 725-731.

Cabrera, M. and K. Jaffe (2007). "An aggregation pheromone modulates lekking behavior in the vector mosquito Aedes aegypti (Diptera: Culicidae)." J. Am. Mosq. Control Assoc. **23**(1): 1-10.
Cammarata, G., D. de Miguel-Perez, A. Russo, A. Peleg, V. Dolo, C. Rolfo and S. Taverna (2022). "Emerging noncoding RNAs contained in extracellular vesicles: rising stars as biomarkers in lung cancer liquid biopsy." Ther. Adv. Med. Oncol. **14**: 17588359221131229.

Campbell, C. L., W. C. t. Black, A. M. Hess and B. D. Foy (2008). "Comparative genomics of small RNA regulatory pathway components in vector mosquitoes." BMC Genomics **9**: 425.

Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." Cell **136**(4): 642-655.

Carvalho, D. O., A. R. McKemey, L. Garziera, R. Lacroix, C. A. Donnelly, L. Alphey, A. Malavasi and M. L. Capurro (2015). "Suppression of a Field Population of Aedes aegypti in Brazil by Sustained Release of Transgenic Male Mosquitoes." PLoS Negl. Trop. Dis. **9**(7): e0003864.
Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, C. Savakis, F. C. Kafatos and A. Crisanti (2000). "Stable germline transformation of the malaria mosquito Anopheles stephensi." Nature **405**(6789): 959-962.

Ceccaldi, R., B. Rondinelli and A. D. D'Andrea (2016). "Repair Pathway Choices and Consequences at the Double-Strand Break." Trends Cell Biol. **26**(1): 52-64.

Cerutti, L., N. Mian and A. Bateman (2000). "Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain." Trends Biochem. Sci. **25**(10): 481-482.

Champer, J., R. Reeves, S. Y. Oh, C. Liu, J. Liu, A. G. Clark and P. W. Messer (2017). "Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations." PLoS Genet. **13**(7): e1006796.

Chapados, B. R., Q. Chai, D. J. Hosfield, J. Qiu, B. Shen and J. A. Tainer (2001). "Structural biochemistry of a type 2 RNase H: RNA primer recognition and removal during DNA replication." J. Mol. Biol. **307**(2): 541-556.

Chaverra-Rodriguez, D., V. M. Macias, G. L. Hughes, S. Pujhari, Y. Suzuki, D. R. Peterson, D. Kim, S. McKeand and J. L. Rasgon (2018). "Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing." Nat. Commun. **9**(1): 3008.
Chiruvella, K. K., Z. Liang and T. E. Wilson (2013). "Repair of double-strand breaks by end joining." Cold Spring Harb. Perspect. Biol. **5**(5): a012757.

Christophers, S. R. (1960). "Aedes aegypti (L.) the yellow fever mosquito: its life history, bionomics and structure." Aëdes aegypti (L. ) the Yellow Fever Mosquito: its Life History, Bionomics and Structure.

Coates, C. J., N. Jasinskiene, L. Miyashiro and A. A. James (1998). "Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3748-3751.

Conant, D., T. Hsiau, N. Rossi, J. Oki, T. Maures, K. Waite, J. Yang, S. Joshi, R. Kelso, K. Holden, B. L. Enzmann and R. Stoner (2022). "Inference of CRISPR Edits from Sanger Trace Data." CRISPR J **5**(1): 123-130.

Crava, C. M., F. S. Varghese, E. Pischedda, R. Halbach, U. Palatini, M. Marconcini, L. Gasmi, S. Redmond, Y. Afrane, D. Ayala, C. Paupy, R. Carballar-Lejarazu, P. Miesen, R. P. van Rij and M. Bonizzoni (2021). "Population genomics in the arboviral vector Aedes aegypti reveals the genomic architecture and evolution of endogenous viral elements." Mol. Ecol. **30**(7): 1594-1611.

Crawford, J. E., J. M. Alves, W. J. Palmer, J. P. Day, M. Sylla, R. Ramasamy, S. N. Surendran, W. C. t. Black, A. Pain and F. M. Jiggins (2017). "Population genomics reveals that an anthropophilic population of Aedes aegypti mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector." BMC Biol. **15**(1): 16.

Crawford, J. E., D. W. Clarke, V. Criswell, M. Desnoyer, D. Cornel, B. Deegan, K. Gong, K. C. Hopkins, P. Howell, J. S. Hyde, J. Livni, C. Behling, R. Benza, W. Chen, K. L. Dobson, C. Eldershaw, D. Greeley, Y. Han, B. Hughes, E. Kakani, J. Karbowski, A. Kitchell, E. Lee, T. Lin, J. Liu, M. Lozano, W. MacDonald, J. W. Mains, M. Metlitz, S. N. Mitchell, D. Moore, J. R. Ohm, K. Parkes, A. Porshnikoff, C. Robuck, M. Sheridan, R. Sobecki, P. Smith, J. Stevenson, J. Sullivan, B. Wasson, A. M. Weakley, M. Wilhelm, J. Won, A. Yasunaga, W. C. Chan, J. Holeman, N. Snoad, L.

Upson, T. Zha, S. L. Dobson, F. S. Mulligan, P. Massaro and B. J. White (2020). "Efficient production of male Wolbachia-infected Aedes aegypti mosquitoes enables large-scale suppression of wild populations." Nat. Biotechnol. **38**(4): 482-492.

Danet, L., G. Beauclair, M. Berthet, G. Moratorio, S. Gracias, F. Tangy, V. Choumet and N. Jouvenet (2019). "Midgut barriers prevent the replication and dissemination of the yellow fever vaccine in Aedes aegypti." PLoS Negl. Trop. Dis. **13**(8): e0007299.

Darricarrère, N., N. Liu, T. Watanabe and H. Lin (2013). "Function of Piwi, a nuclear Piwi/Argonaute protein, is independent of its slicer activity." Proc. Natl. Acad. Sci. U. S. A. **110**(4): 1297-1302.

de Almeida, J. P., E. R. Aguiar, J. N. Armache, R. P. Olmo and J. T. Marques (2021). "The virome of vector mosquitoes." Curr. Opin. Virol. **49**: 7-12.

Deehan, M., W. Lin, B. Blum, A. Emili and H. Frydman (2021). "Intracellular Density of Wolbachia Is Mediated by Host Autophagy and the Bacterial Cytoplasmic Incompatibility Gene cifB in a Cell Type-Dependent Manner in Drosophila melanogaster." MBio **12**(1).

Degner, E. C. and L. C. Harrington (2016). "Polyandry Depends on Postmating Time Interval in the Dengue Vector Aedes aegypti." Am. J. Trop. Med. Hyg. **94**(4): 780-785.

Deredec, A., A. Burt and H. C. J. Godfray (2008). "The population genetics of using homing endonuclease genes in vector and pest management." Genetics **179**(4): 2013-2026.

Derryberry, O. M. and F. E. Gartrell (1952). "Trends in malaria control program of the Tennessee Valley Authority." Am. J. Trop. Med. Hyg. **1**(3): 500-507.

Dietrich, I., S. Jansen, G. Fall, S. Lorenzen, M. Rudolf, K. Huber, A. Heitmann, S. Schicht, E. H. Ndiaye, M. Watson, I. Castelli, B. Brennan, R. M. Elliott, M. Diallo, A. A. Sall, A.-B. Failloux, E. Schnettler, A. Kohl and S. C. Becker (2017). "RNA Interference Restricts Rift Valley Fever Virus in Multiple Insect Systems." mSphere **2**(3).

Dong, S., J. Lin, N. L. Held, R. J. Clem, A. L. Passarelli and A. W. E. Franz (2015). "Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti." PLoS One **10**(3): e0122353.

Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science **346**(6213): 1258096.

Drinnenberg, I. A., D. E. Weinberg, K. T. Xie, J. P. Mower, K. H. Wolfe, G. R. Fink and D. P. Bartel (2009). "RNAi in budding yeast." Science **326**(5952): 544-550.

El Chamy, L., V. Leclerc, I. Caldelari and J.-M. Reichhart (2008). "Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll." Nat. Immunol. **9**(10): 1165-1170.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes Dev. **15**(2): 188-200.

Elissa, N., J. Mouchet, F. Riviere, J. Y. Meunier and K. Yao (1993). "Resistance of Anopheles gambiae s.s. to pyrethroids in Côte d'Ivoire." Ann. Soc. Belg. Med. Trop. **73**(4): 291-294.

Evans, B. R., P. Kotsakiozi, A. L. Costa-da-Silva, R. S. Ioshino, L. Garziera, M. C. Pedrosa, A. Malavasi, J. F. Virginio, M. L. Capurro and J. R. Powell (2019). "Transgenic Aedes aegypti Mosquitoes Transfer Genes into a Natural Population." Sci. Rep. **9**(1): 13047.

Fawaz, E. Y., S. A. Allan, U. R. Bernier, P. J. Obenauer and J. W. Diclaro, 2nd (2014). "Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of Aedes aegypti." J. Vector Ecol. **39**(2): 347-354.

Ferguson, N. M., D. T. H. Kien, H. Clapham, R. Aguas, V. T. Trung, T. N. B. Chau, J. Popovici, P. A. Ryan, S. L. O'Neill, E. A. McGraw, V. T. Long, L. T. Dui, H. L. Nguyen, N. V. V. Chau, B. Wills and C. P. Simmons (2015). "Modeling the impact on virus transmission of Wolbachia-mediated blocking of dengue virus infection of Aedes aegypti." Sci. Transl. Med. **7**(279): 279ra237.
Ferroni, E., T. Jefferson and G. Gachelin (2012). "Angelo Celli and research on the prevention of malaria in Italy a century ago." J. R. Soc. Med. **105**(1): 35-40.

Frank, F., N. Sonenberg and B. Nagar (2010). "Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2." Nature **465**(7299): 818-822.

Frank, J. A. and C. Feschotte (2017). "Co-option of endogenous viral sequences for host cell function." Curr. Opin. Virol. **25**: 81-89.

Franz, A. W. E., A. M. Kantor, A. L. Passarelli and R. J. Clem (2015). "Tissue Barriers to Arbovirus Infection in Mosquitoes." Viruses **7**(7): 3741-3767.

Franz, A. W. E., I. Sanchez-Vargas, Z. N. Adelman, C. D. Blair, B. J. Beaty, A. A. James and K. E. Olson (2006). "Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **103**(11): 4198-4203.

Fredericks, A. C., T. A. Russell, L. E. Wallace, A. D. Davidson, A. Fernandez-Sesma and K. Maringer (2019). "Aedes aegypti (Aag2)-derived clonal mosquito cell lines reveal the effects of pre-existing persistent infection with the insect-specific bunyavirus Phasi Charoen-like virus on arbovirus replication." PLoS Negl. Trop. Dis. **13**(11): e0007346.

Gainetdinov, I., C. Colpan, A. Arif, K. Cecchini and P. D. Zamore (2018). "A Single Mechanism of Biogenesis, Initiated and Directed by PIWI Proteins, Explains piRNA Production in Most Animals." Mol. Cell **71**(5): 775-790.e775.

Galton, R., K. Fejes-Toth and M. E. Bronner (2022). "Co-option of the piRNA pathway to regulate neural crest specification." Science Advances **8**(32): eabn1441.

Gan, S. J., Y. Q. Leong, M. F. H. Bin Barhanuddin, S. T. Wong, S. F. Wong, J. W. Mak and R. B. Ahmad (2021). "Dengue fever and insecticide resistance in Aedes mosquitoes in Southeast Asia: a review." Parasit. Vectors **14**(1): 315.

Gantz, V. M., N. Jasinskiene, O. Tatarenkova, A. Fazekas, V. M. Macias, E. Bier and A. A. James (2015). "Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi." Proc. Natl. Acad. Sci. U. S. A. **112**(49): E6736-6743.
Geoghegan, V., K. Stainton, S. M. Rainey, T. H. Ant, A. A. Dowle, T. Larson, S. Hester, P. D. Charles, B. Thomas and S. P. Sinkins (2017). "Perturbed cholesterol and vesicular trafficking associated with dengue blocking in Wolbachia-infected Aedes aegypti cells." Nat. Commun. **8**(1): 526.

Girardi, E., P. Miesen, B. Pennings, L. Frangeul, M.-C. Saleh and R. P. van Rij (2017). "Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in Aedes aegypti." Nucleic Acids Res. **45**(8): 4881-4892.

Gloria-Soria, A., T. G. Chiodo and J. R. Powell (2018). "Lack of Evidence for Natural Wolbachia Infections in Aedes aegypti (Diptera: Culicidae)." J. Med. Entomol. **55**(5): 1354-1356.

Göertz, G. P., J. J. Fros, P. Miesen, C. B. F. Vogels, M. L. van der Bent, C. Geertsema, C. J. M. Koenraadt, R. P. van Rij, M. M. van Oers and G. P. Pijlman (2016). "Noncoding Subgenomic Flavivirus RNA Is Processed by the Mosquito RNA Interference Machinery and Determines West Nile Virus Transmission by Culex pipiens Mosquitoes." J. Virol. **90**(22): 10145-10159.

Goic, B., K. A. Stapleford, L. Frangeul, A. J. Doucet, V. Gausson, H. Blanc, N. Schemmel-Jofre, G. Cristofari, L. Lambrechts, M. Vignuzzi and M.-C. Saleh (2016). "Virus-derived DNA drives mosquito vector tolerance to arboviral infection." Nat. Commun. **7**: 12410.

Goic, B., N. Vodovar, J. A. Mondotte, C. Monot, L. Frangeul, H. Blanc, V. Gausson, J. Vera-Otarola, G. Cristofari and M.-C. Saleh (2013). "RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila." Nat. Immunol. **14**(4): 396-403.

Goriaux, C., S. Desset, Y. Renaud, C. Vaury and E. Brasset (2014). "Transcriptional properties and splicing of the flamenco piRNA cluster." EMBO Rep. **15**(4): 411-418.

Grossman, G. L., C. S. Rafferty, J. R. Clayton, T. K. Stevens, O. Mukabayire and M. Q. Benedict (2001). "Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element." Insect Mol. Biol. **10**(6): 597-604.

Grubaugh, N. D., J. Weger-Lucarelli, R. A. Murrieta, J. R. Fauver, S. M. Garcia-Luna, A. N. Prasad, W. C. t. Black and G. D. Ebel (2016). "Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching." Cell Host Microbe **19**(4): 481-492.

Gunawardane, L. S., K. Saito, K. M. Nishida, K. Miyoshi, Y. Kawamura, T. Nagami, H. Siomi and M. C. Siomi (2007). "A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila." Science **315**(5818): 1587-1590.

Gwadz, R. W., G. B. Craig, Jr. and W. A. Hickey (1971). "Female sexual behavior as the mechanism rendering Aedes aegypti refractory to insemination." Biol. Bull. **140**(2): 201-214.

Halbach, R., P. Miesen, J. Joosten, E. Taşköprü, I. Rondeel, B. Pennings, C. B. F. Vogels, S. H. Merkling, C. J. Koenraadt, L. Lambrechts and R. P. van Rij (2020). "A satellite repeat-derived piRNA controls embryonic development of Aedes." Nature.

Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, A. Burt, N. Windbichler, A. Crisanti and T. Nolan (2016). "A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae." Nat. Biotechnol. **34**(1): 78-83.

Hammond, A., P. Pollegioni, T. Persampieri, A. North, R. Minuz, A. Trusso, A. Bucci, K. Kyrou, I. Morianou, A. Simoni, T. Nolan, R. Müller and A. Crisanti (2021). "Gene-drive suppression of mosquito populations in large cages as a bridge between lab and field." Nat. Commun. **12**(1): 4589.
Hammond, A. M., K. Kyrou, M. Bruttini, A. North, R. Galizi, X. Karlsson, N. Kranjc, F. M. Carpi, R. D'Aurizio, A. Crisanti and T. Nolan (2017). "The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito." PLoS Genet. **13**(10): e1007039.
Hammond, S. M., E. Bernstein, D. Beach and G. J. Hannon (2000). "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells." Nature **404**(6775): 293-296.
Han, M. E. (2017). The piRNA System in Aedes aegypti. Ph.D. Ph.D. Thesis, University of California, Riverside.

Handler, A. M. (2001). "A current perspective on insect gene transformation." Insect Biochem. Mol. Biol. **31**(2): 111-128.

Harris, A. F., A. R. McKemey, D. Nimmo, Z. Curtis, I. Black, S. A. Morgan, M. N. Oviedo, R. Lacroix, N. Naish, N. I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C. A. Donnelly, W. D. Petrie and L. Alphey

(2012). "Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes." Nat. Biotechnol. **30**(9): 828-830.

Harris, A. F., D. Nimmo, A. R. McKemey, N. Kelly, S. Scaife, C. A. Donnelly, C. Beech, W. D. Petrie and L. Alphey (2011). "Field performance of engineered male mosquitoes." Nat. Biotechnol. **29**(11): 1034-1037.

Hartberg, W. K. (1971). "Observations on the mating behaviour of Aedes aegypti in nature." Bull. World Health Organ. **45**(6): 847-850.

Hayashi, R., J. Schnabl, D. Handler, F. Mohn, S. L. Ameres and J. Brennecke (2016). "Genetic and mechanistic diversity of piRNA 3′-end formation." Nature **539**(7630): 588-592.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill and K. N. Johnson (2008). "Wolbachia and virus protection in insects." Science **322**(5902): 702.

Hess, A. M., A. N. Prasad, A. Ptitsyn, G. D. Ebel, K. E. Olson, C. Barbacioru, C. Monighetti and C. L. Campbell (2011). "Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense." BMC Microbiol. **11**: 45.

Higashitani, Y. and K. Horie (2023). "Long-read sequence analysis of MMEJ-mediated CRISPR genome editing reveals complex on-target vector insertions that may escape standard PCR-based quality control." Sci. Rep. **13**(1): 11652.

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, H. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli and S. L. O'Neill (2011). "Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission." Nature **476**(7361): 454-457.

Holcomb, K. (2022). "Worst-ever U.S. West Nile virus outbreak potentially linked to a wetter-than-average 2021 Southwest monsoon."

Huang, Y., Z. Liu and Y. S. Rong (2016). "Genome Editing: From Drosophila to Non-Model Insects and Beyond." J. Genet. Genomics **43**(5): 263-272.

Hussain, M., S. Torres, E. Schnettler, A. Funk, A. Grundhoff, G. P. Pijlman, A. A. Khromykh and S. Asgari (2012). "West Nile virus encodes a microRNA-like small RNA in the 3′ untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells." Nucleic Acids Res. **40**(5): 2210-2223.

Hutvagner, G. and M. J. Simard (2008). "Argonaute proteins: key players in RNA silencing." Nat. Rev. Mol. Cell Biol. **9**(1): 22-32.

Ingolia, N. T., G. A. Brar, S. Rouskin, A. M. McGeachy and J. S. Weissman (2012). "The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments." Nat. Protoc. **7**(8): 1534-1550.

Jangir, P. K. and A. Prasad (2022). "Spatial distribution of insecticide resistance and susceptibility in Aedes aegypti and Aedes albopictus in India." Int. J. Trop. Insect Sci. **42**(2): 1019-1044.

Jasinskiene, N., C. J. Coates, M. Q. Benedict, A. J. Cornel, C. S. Rafferty, A. A. James and F. H. Collins (1998). "Stable transformation of the yellow fever mosquito, Aedes aegypti, with the Hermes element from the housefly." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3743-3747.

Jehn, J., D. Gebert, F. Pipilescu, S. Stern, J. S. T. Kiefer, C. Hewel and D. Rosenkranz (2018). "PIWI genes and piRNAs are ubiquitously expressed in mollusks and show patterns of lineage-specific adaptation." Commun Biol **1**: 137.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science **337**(6096): 816-821.

Joosten, J., P. Miesen, E. Taşköprü, B. Pennings, P. W. T. C. Jansen, M. A. Huynen, M. Vermeulen and R. P. Van Rij (2019). "The Tudor protein Veneno assembles the ping-pong amplification complex that produces viral piRNAs in Aedes mosquitoes." Nucleic Acids Res. **47**(5): 2546-2559.

Kakumani, P. K., S. S. Ponia, R. K. S, V. Sood, M. Chinnappan, A. C. Banerjea, G. R. Medigeshi, P. Malhotra, S. K. Mukherjee and R. K. Bhatnagar (2013). "Role of RNA interference (RNAi) in dengue virus replication and identification of NS4B as an RNAi suppressor." J. Virol. **87**(16): 8870-8883.

Kandul, N. P., J. Liu, J. B. Bennett, J. M. Marshall and O. S. Akbari (2021). "A confinable home-and-rescue gene drive for population modification." Elife **10**.

Kandul, N. P., J. Liu, H. M. Sanchez C, S. L. Wu, J. M. Marshall and O. S. Akbari (2019). "Transforming insect population control with precision guided sterile males with demonstration in flies." Nat. Commun. **10**(1): 84.

Katzourakis, A. and R. J. Gifford (2010). "Endogenous viral elements in animal genomes." PLoS Genet. **6**(11): e1001191.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat. Rev. Mol. Cell Biol. **10**(2): 126-139.

Kistler, K. E., L. B. Vosshall and B. J. Matthews (2015). "Genome engineering with CRISPR-Cas9 in the mosquito Aedes aegypti." Cell Rep. **11**(1): 51-60.

Kittayapong, P. (2021). Combined sterile insect technique and incompatible insect technique: concept, study design, experience and lessons learned from a pilot suppression trial in Thailand. Area-Wide Integrated Pest Management, CRC Press**:** 405-432.

Kittayapong, P., S. Ninphanomchai, W. Limohpasmanee, C. Chansang, U. Chansang and P. Mongkalangoon (2019). "Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress Aedes aegypti vector populations in semi-rural settings in Thailand." PLoS Negl. Trop. Dis. **13**(10): e0007771.

Kiuchi, T., H. Koga, M. Kawamoto, K. Shoji, H. Sakai, Y. Arai, G. Ishihara, S. Kawaoka, S. Sugano, T. Shimada, Y. Suzuki, M. G. Suzuki and S. Katsuma (2014). "A single female-specific piRNA is the primary determiner of sex in the silkworm." Nature **509**(7502): 633-636.

Klattenhoff, C., H. Xi, C. Li, S. Lee, J. Xu, J. S. Khurana, F. Zhang, N. Schultz, B. S. Koppetsch, A. Nowosielska, H. Seitz, P. D. Zamore, Z. Weng and W. E. Theurkauf (2009). "The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters." Cell **138**(6): 1137-1149.

Knipling, E. F. (1979). The Basic Principles of Insect Population Suppression and Management, U.S. Department of Agriculture.

Kokoza, V., A. Ahmed, E. A. Wimmer and A. S. Raikhel (2001). "Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[3xP3-EGFP afm]." Insect Biochem. Mol. Biol. **31**(12): 1137-1143.

Koopal, B., S. K. Mutte and D. C. Swarts (2023). "A long look at short prokaryotic Argonautes." Trends Cell Biol. **33**(7): 605-618.

Kosicki, M., K. Tomberg and A. Bradley (2018). "Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements." Nat. Biotechnol. **36**(8): 765-771.

Kyrou, K., A. M. Hammond, R. Galizi, N. Kranjc, A. Burt, A. K. Beaghton, T. Nolan and A. Crisanti (2018). "A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes." Nat. Biotechnol. **36**(11): 1062-1066.

Lambrechts, L. and W. Scott Thomas (2009). "Mode of transmission and the evolution of arbovirus virulence in mosquito vectors." Proceedings of the Royal Society B: Biological Sciences **276**(1660): 1369-1378.

Lan, Q. and A. M. Fallon (1990). "Small heat shock proteins distinguish between two mosquito species and confirm identity of their cell lines." Am. J. Trop. Med. Hyg. **43**(6): 669-676.

Laven, H., J. Cousserans and G. Guille (1972). "Eradicating mosquitoes using translocations: a first field experiment." Nature **236**(5348): 456-457.

Le Thomas, A., A. K. Rogers, A. Webster, G. K. Marinov, S. E. Liao, E. M. Perkins, J. K. Hur, A. A. Aravin and K. F. Tóth (2013). "Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state." Genes Dev. **27**(4): 390-399.

Le Thomas, A., E. Stuwe, S. Li, J. Du, G. Marinov, N. Rozhkov, Y.-C. A. Chen, Y. Luo, R. Sachidanandam, K. F. Toth, D. Patel and A. A. Aravin (2014). "Transgenerationally inherited piRNAs trigger piRNA biogenesis by changing the chromatin of piRNA clusters and inducing precursor processing." Genes Dev. **28**(15): 1667-1680.

Lee, Y., H. Schmidt, T. C. Collier, W. R. Conner, M. J. Hanemaaijer, M. Slatkin, J. M. Marshall, J. C. Chiu, C. T. Smartt, G. C. Lanzaro, F. S. Mulligan and A. J. Cornel (2019). "Genome-wide divergence among invasive populations of Aedes aegypti in California." BMC Genomics **20**(1): 204.

Leftwich, P. T., L. G. Spurgin, T. Harvey-Samuel, C. J. E. Thomas, L. C. Paladino, M. P. Edgington and L. Alphey (2021). "Genetic pest management and the background genetics of release strains." Philos. Trans. R. Soc. Lond. B Biol. Sci. **376**(1818): 20190805.

Lemaitre, B. and J. Hoffmann (2007). "The host defense of Drosophila melanogaster." Annu. Rev. Immunol. **25**: 697-743.

LePage, D. P., J. A. Metcalf, S. R. Bordenstein, J. On, J. I. Perlmutter, J. D. Shropshire, E. M. Layton, L. J. Funkhouser-Jones, J. F. Beckmann and S. R. Bordenstein (2017). "Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility." Nature **543**(7644): 243-247.

Lewis, S. H., K. A. Quarles, Y. Yang, M. Tanguy, L. Frézal, S. A. Smith, P. P. Sharma, R. Cordaux, C. Gilbert, I. Giraud, D. H. Collins, P. D. Zamore, E. A. Miska, P. Sarkies and F. M. Jiggins (2018). "Pan-arthropod analysis reveals somatic piRNAs as an ancestral defence against transposable elements." Nat Ecol Evol **2**(1): 174-181.

Lewis, S. H., H. Salmela and D. J. Obbard (2016). "Duplication and Diversification of Dipteran Argonaute Genes, and the Evolutionary Divergence of Piwi and Aubergine." Genome Biol. Evol. **8**(3): 507-518.

Lewis, S. H., C. L. Webster, H. Salmela and D. J. Obbard (2016). "Repeated Duplication of Argonaute2 Is Associated with Strong Selection and Testis Specialization in Drosophila." Genetics.

Li, M., M. Bui, T. Yang, C. S. Bowman, B. J. White and O. S. Akbari (2017). "Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **114**(49): E10540-E10549.

Li, M., T. Yang, M. Bui, S. Gamez, T. Wise, N. P. Kandul, J. Liu, L. Alcantara, H. Lee, J. R. Edula, R. Raban, Y. Zhan, Y. Wang, N. DeBeaubien, J. Chen, H. M. Sánchez C, J. B. Bennett, I. Antoshechkin, C. Montell, J. M. Marshall and O. S. Akbari (2021). "Suppressing mosquito populations with precision guided sterile males." Nat. Commun. **12**(1): 5374.

Li, M., T. Yang, N. P. Kandul, M. Bui, S. Gamez, R. Raban, J. Bennett, H. M. Sánchez C, G. C. Lanzaro, H. Schmidt, Y. Lee, J. M. Marshall and O. S. Akbari (2020). "Development of a confinable gene drive system in the human disease vector Aedes aegypti." Elife **9**.

Li, W.-X., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. A. Brydon, K. L. Johnson, A. García-Sastre, L. A. Ball, P. Palese and S.-W. Ding (2004). "Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing." Proc. Natl. Acad. Sci. U. S. A. **101**(5): 1350-1355.

Li, X. Z., C. K. Roy, X. Dong, E. Bolcun-Filas, J. Wang, B. W. Han, J. Xu, M. J. Moore, J. C. Schimenti, Z. Weng and P. D. Zamore (2013). "An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes." Mol. Cell **50**(1): 67-81.

Lindquist, D. A., M. Abusowa and M. J. Hall (1992). "The New World screwworm fly in Libya: a review of its introduction and eradication." Med. Vet. Entomol. **6**(1): 2-8.

Liu-Helmersson, J., H. Stenlund, A. Wilder-Smith and J. Rocklöv (2014). "Vectorial capacity of Aedes aegypti: effects of temperature and implications for global dengue epidemic potential." PLoS One **9**(3): e89783.

Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J.-J. Song, S. M. Hammond, L. Joshua-Tor and G. J. Hannon (2004). "Argonaute2 is the catalytic engine of mammalian RNAi." Science **305**(5689): 1437-1441.

Liu, Q. and Z. Paroo (2010). "Biochemical principles of small RNA pathways." Annu. Rev. Biochem. **79**: 295-319.

Lobo, N. F., A. Hua-Van, X. Li, B. M. Nolen and M. J. Fraser, Jr. (2002). "Germ line transformation of the yellow fever mosquito, Aedes aegypti, mediated by transpositional insertion of a piggyBac vector." Insect Mol. Biol. **11**(2): 133-139.

Lounibos, L. P. (1981). "Habitat segregation among African treehole mosquitoes." Ecol. Entomol. **6**(2): 129-154.

Lu, H.-L., S. Tanguy, C. Rispe, J.-P. Gauthier, T. Walsh, K. Gordon, O. Edwards, D. Tagu, C.-C. Chang and S. Jaubert-Possamai (2011). "Expansion of Genes Encoding piRNA-Associated Argonaute Proteins in the Pea Aphid: Diversification of Expression Profiles in Different Plastic Morphs." PLoS One **6**(12): e28051.

Ma, J.-B., Y.-R. Yuan, G. Meister, Y. Pei, T. Tuschl and D. J. Patel (2005). "Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein." Nature **434**(7033): 666-670.

Malone, C. D., J. Brennecke, M. Dus, A. Stark, W. R. McCombie, R. Sachidanandam and G. J. Hannon (2009). "Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary." Cell **137**(3): 522-535.

Marconcini, M., L. Hernandez, G. Iovino, V. Houé, F. Valerio, U. Palatini, E. Pischedda, J. E. Crawford, B. J. White, T. Lin, R. Carballar-Lejarazu, L. Ometto, F. Forneris, A.-B. Failloux and M. Bonizzoni (2019). "Polymorphism analyses and protein modelling inform on functional specialization of Piwi clade genes in the arboviral vector Aedes albopictus." PLoS Negl. Trop. Dis. **13**(12): e0007919.

Marconcini, M., E. Pischedda, V. Houé, U. Palatini, N. Lozada-Chávez, D. Sogliani, A.-B. Failloux and M. Bonizzoni (2021). "Profile of Small RNAs, vDNA Forms and Viral Integrations in Late Chikungunya Virus Infection of Aedes albopictus Mosquitoes." Viruses **13**(4): 553.

Martinez, J. and T. Tuschl (2004). "RISC is a 5′ phosphomonoester-producing RNA endonuclease." Genes Dev. **18**(9): 975-980.

Matsumoto, N., H. Nishimasu, K. Sakakibara, K. M. Nishida, T. Hirano, R. Ishitani, H. Siomi, M. C. Siomi and O. Nureki (2016). "Crystal Structure of Silkworm PIWI-Clade Argonaute Siwi Bound to piRNA." Cell **167**(2): 484-497.e489.

Matthews, B. J., O. Dudchenko, S. B. Kingan, S. Koren, I. Antoshechkin, J. E. Crawford, W. J. Glassford, M. Herre, S. N. Redmond, N. H. Rose, G. D. Weedall, Y. Wu, S. S. Batra, C. A. Brito-Sierra, S. D. Buckingham, C. L. Campbell, S. Chan, E. Cox, B. R. Evans, T. Fansiri, I. Filipović, A. Fontaine, A. Gloria-Soria, R. Hall, V. S. Joardar, A. K. Jones, R. G. G. Kay, V. K. Kodali, J. Lee, G. J. Lycett, S. N. Mitchell, J. Muehling, M. R. Murphy, A. D. Omer, F. A. Partridge, P. Peluso, A. P.

Aiden, V. Ramasamy, G. Rašić, S. Roy, K. Saavedra-Rodriguez, S. Sharan, A. Sharma, M. L. Smith, J. Turner, A. M. Weakley, Z. Zhao, O. S. Akbari, W. C. t. Black, H. Cao, A. C. Darby, C. A. Hill, J. S. Johnston, T. D. Murphy, A. S. Raikhel, D. B. Sattelle, I. V. Sharakhov, B. J. White, L. Zhao, E. L. Aiden, R. S. Mann, L. Lambrechts, J. R. Powell, M. V. Sharakhova, Z. Tu, H. M. Robertson, C. S. McBride, A. R. Hastie, J. Korlach, D. E. Neafsey, A. M. Phillippy and L. B. Vosshall (2018). "Improved reference genome of Aedes aegypti informs arbovirus vector control." Nature **563**(7732): 501-507.

McVey, M. and S. E. Lee (2008). "MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings." Trends Genet. **24**(11): 529-538.

Meister, G. (2013). "Argonaute proteins: functional insights and emerging roles." Nat. Rev. Genet. **14**(7): 447-459.

Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl (2004). "Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs." Mol. Cell **15**(2): 185-197.
Merkling, S. H. and R. P. van Rij (2013). "Beyond RNAi: antiviral defense strategies in Drosophila and mosquito." J. Insect Physiol. **59**(2): 159-170.

Messina, J. P., O. J. Brady, N. Golding, M. U. G. Kraemer, G. R. W. Wint, S. E. Ray, D. M. Pigott, F. M. Shearer, K. Johnson, L. Earl, L. B. Marczak, S. Shirude, N. Davis Weaver, M. Gilbert, R. Velayudhan, P. Jones, T. Jaenisch, T. W. Scott, R. C. Reiner, Jr. and S. I. Hay (2019). "The current and future global distribution and population at risk of dengue." Nat Microbiol **4**(9): 1508-1515.

Metzger, M. E., M. Hardstone Yoshimizu, K. A. Padgett, R. Hu and V. L. Kramer (2017). "Detection and Establishment of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) Mosquitoes in California, 2011-2015." J. Med. Entomol. **54**(3): 533-543.

Miesen, P., E. Girardi and R. P. van Rij (2015). "Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in Aedes aegypti mosquito cells." Nucleic Acids Res. **43**(13): 6545-6556.

Miesen, P., A. Ivens, A. H. Buck and R. P. van Rij (2016). "Small RNA Profiling in Dengue Virus 2-Infected Aedes Mosquito Cells Reveals Viral piRNAs and Novel Host miRNAs." PLoS Negl. Trop. Dis. **10**(2): e0004452.

Miesen, P., J. Joosten and R. P. van Rij (2016). "PIWIs Go Viral: Arbovirus-Derived piRNAs in Vector Mosquitoes." PLoS Pathog. **12**(12): e1006017.

Mohn, F., D. Handler and J. Brennecke (2015). "Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis." Science **348**(6236): 812-817.

Mohn, F., G. Sienski, D. Handler and J. Brennecke (2014). "The rhino-deadlock-cutoff complex licenses noncanonical transcription of dual-strand piRNA clusters in Drosophila." Cell **157**(6): 1364-1379.

Mondotte, J. A., V. Gausson, L. Frangeul, Y. Suzuki, M. Vazeille, V. Mongelli, H. Blanc, A.-B. Failloux and M.-C. Saleh (2020). "Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects." Cell Rep. **33**(11): 108506.

Morazzani, E. M., M. R. Wiley, M. G. Murreddu, Z. N. Adelman and K. M. Myles (2012). "Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma." PLoS Pathog. **8**(1): e1002470.

Moyes, C. L., J. Vontas, A. J. Martins, L. C. Ng, S. Y. Koou, I. Dusfour, K. Raghavendra, J. Pinto, V. Corbel, J.-P. David and D. Weetman (2017). "Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans." PLoS Negl. Trop. Dis. **11**(7): e0005625.

Murzin, A. G. (1993). "OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences." EMBO J. **12**(3): 861-867.

Myles, K. M., D. J. Pierro and K. E. Olson (2003). "Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti." J. Virol. **77**(16): 8872-8881.

Nájera, J. A., M. González-Silva and P. L. Alonso (2011). "Some lessons for the future from the Global Malaria Eradication Programme (1955-1969)." PLoS Med. **8**(1): e1000412.

Nakanishi, K., D. E. Weinberg, D. P. Bartel and D. J. Patel (2012). "Structure of yeast Argonaute with guide RNA." Nature **486**(7403): 368-374.

Navarro-Payá, D., I. Flis, M. A. E. Anderson, P. Hawes, M. Li, O. S. Akbari, S. Basu and L. Alphey (2020). "Targeting female flight for genetic control of mosquitoes." PLoS Negl. Trop. Dis. **14**(12): e0008876.

Ngugi, H. N., F. M. Mutuku, B. A. Ndenga, P. S. Musunzaji, J. O. Mbakaya, P. Aswani, L. W. Irungu, D. Mukoko, J. Vulule, U. Kitron and A. D. LaBeaud (2017). "Characterization and productivity profiles of Aedes aegypti (L.) breeding habitats across rural and urban landscapes in western and coastal Kenya." Parasit. Vectors **10**(1): 331.

Nishida, K. M., K. Saito, T. Mori, Y. Kawamura, T. Nagami-Okada, S. Inagaki, H. Siomi and M. C. Siomi (2007). "Gene silencing mechanisms mediated by Aubergine piRNA complexes in Drosophila male gonad." RNA **13**(11): 1911-1922.

Nosrat, C., J. Altamirano, A. Anyamba, J. M. Caldwell, R. Damoah, F. Mutuku, B. Ndenga and A. D. LaBeaud (2021). "Impact of recent climate extremes on mosquito-borne disease transmission in Kenya." PLoS Negl. Trop. Dis. **15**(3): e0009182.

O'Brochta, D. A. and P. W. Atkinson (1996). "Transposable elements and gene transformation in non-drosophilid insects." Insect Biochem. Mol. Biol. **26**(8-9): 739-753.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin and S. L. Dobson (2012). "Open release of male mosquitoes infected with a wolbachia biopesticide: field performance and infection containment." PLoS Negl. Trop. Dis. **6**(11): e1797.

Olmo, R. P., A. G. A. Ferreira, T. C. Izidoro-Toledo, E. R. G. R. Aguiar, I. J. S. de Faria, K. P. R. de Souza, K. P. Osório, L. Kuhn, P. Hammann, E. G. de Andrade, Y. M. Todjro, M. N. Rocha, T. H. J. F. Leite, S. C. G. Amadou, J. N. Armache, S. Paro, C. D. de Oliveira, F. D. Carvalho, L. A. Moreira, E. Marois, J.-L. Imler and J. T. Marques (2018). "Control of dengue virus in the midgut of Aedes aegypti by ectopic expression of the dsRNA-binding protein Loqs2." Nat Microbiol **3**(12): 1385-1393.

Olmo, R. P., Y. M. H. Todjro, E. R. G. R. Aguiar, J. P. P. de Almeida, F. V. Ferreira, J. N. Armache, I. J. S. de Faria, A. G. A. Ferreira, S. C. G. Amadou, A. T. S. Silva, K. P. R. de Souza, A. P. P. Vilela, A. Babarit, C. H. Tan, M. Diallo, A. Gaye, C. Paupy, J. Obame-Nkoghe, T. M. Visser, C. J. M. Koenraadt, M. A. Wongsokarijo, A. L. C. Cruz, M. T. Prieto, M. C. P. Parra, M. L. Nogueira, V. Avelino-Silva, R. N. Mota, M. A. Z. Borges, B. P. Drumond, E. G. Kroon, M. Recker, L. Sedda, E. Marois, J.-L. Imler and J. T. Marques (2023). "Mosquito vector competence for dengue is modulated by insect-specific viruses." Nat Microbiol **8**(1): 135-149.

Ophinni, Y., U. Palatini, Y. Hayashi and N. F. Parrish (2019). "piRNA-Guided CRISPR-like Immunity in Eukaryotes." Trends Immunol. **40**(11): 998-1010.

Organization, W. H. (1964). "Genetics of Vectors and Insecticide Resistance: Report of a WHO Scientific Group [meeting held in Geneva from 5 to 9 August 1963]."

Ouédraogo, W. M., K. H. Toé, A. Sombié, M. Viana, C. Bougouma, A. Sanon, D. Weetman, P. J. McCall, H. Kanuka and A. Badolo (2022). "Impact of physicochemical parameters of Aedes aegypti breeding habitats on mosquito productivity and the size of emerged adult mosquitoes in Ouagadougou City, Burkina Faso." Parasit. Vectors **15**(1): 478.

Overcash, J. and Z. N. Adelman (2016). Chapter One - Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. Advances in Insect Physiology. A. S. Raikhel, Academic Press. **51:** 1-35.

Palatini, U., P. Miesen, R. Carballar-Lejarazu, L. Ometto, E. Rizzo, Z. Tu, R. P. van Rij and M. Bonizzoni (2017). "Comparative genomics shows that viral integrations are abundant and express piRNAs in the arboviral vectors Aedes aegypti and Aedes albopictus." BMC Genomics **18**(1): 512.

Pane, A., P. Jiang, D. Y. Zhao, M. Singh and T. Schüpbach (2011). "The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline." EMBO J. **30**(22): 4601-4615.

Paradkar, P. N., J.-B. Duchemin, R. Voysey and P. J. Walker (2014). "Dicer-2-dependent activation of Culex Vago occurs via the TRAF-Rel2 signaling pathway." PLoS Negl. Trop. Dis. **8**(4): e2823.

Paradkar, P. N., L. Trinidad, R. Voysey, J.-B. Duchemin and P. J. Walker (2012). "Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway." Proc. Natl. Acad. Sci. U. S. A. **109**(46): 18915-18920.

Park, S. H., M. Cao and G. Bao (2023). "Detection and quantification of unintended large on-target gene modifications due to CRISPR/Cas9 editing." Current Opinion in Biomedical Engineering **28**: 100478.

Parker, J. S., S. M. Roe and D. Barford (2005). "Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex." Nature **434**(7033): 663-666.

Petit, M., V. Mongelli, L. Frangeul, H. Blanc, F. Jiggins and M.-C. Saleh (2016). "piRNA pathway is not required for antiviral defense in Drosophila melanogaster." Proc. Natl. Acad. Sci. U. S. A. **113**(29): E4218-4227.

Phuc, H. K., M. H. Andreasen, R. S. Burton, C. Vass, M. J. Epton, G. Pape, G. Fu, K. C. Condon, S. Scaife, C. A. Donnelly, P. G. Coleman, H. White-Cooper and L. Alphey (2007). "Late-acting dominant lethal genetic systems and mosquito control." BMC Biol. **5**: 11.

Pless, E., A. Gloria-Soria, B. R. Evans, V. Kramer, B. G. Bolling, W. J. Tabachnick and J. R. Powell (2017). "Multiple introductions of the dengue vector, Aedes aegypti, into California." PLoS Negl. Trop. Dis. **11**(8): e0005718.

Poirier, E. Z., B. Goic, L. Tomé-Poderti, L. Frangeul, J. Boussier, V. Gausson, H. Blanc, T. Vallet, H. Loyd, L. I. Levi, S. Lanciano, C. Baron, S. H. Merkling, L. Lambrechts, M. Mirouze, S. Carpenter, M. Vignuzzi and M.-C. Saleh (2018). "Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects." Cell Host Microbe **23**(3): 353-365.e358.

Powell, J. R. and W. J. Tabachnick (2013). "History of domestication and spread of Aedes aegypti--a review." Mem. Inst. Oswaldo Cruz **108 Suppl 1**: 11-17.

Price, T. A. R., N. Windbichler, R. L. Unckless, A. Sutter, J.-N. Runge, P. A. Ross, A. Pomiankowski, N. L. Nuckolls, C. Montchamp-Moreau, N. Mideo, O. Y. Martin, A. Manser, M. Legros, A. M. Larracuente, L. Holman, J. Godwin, N. Gemmell, C. Courret, A. Buchman, L. G. Barrett and A. K. Lindholm (2020). "Resistance to natural and synthetic gene drive systems." J. Evol. Biol. **33**(10): 1345-1360.

Qu, J., V. Betting, R. van Iterson, F. M. Kwaschik and R. P. van Rij (2023). "Chromatin profiling identifies transcriptional readthrough as a conserved mechanism for piRNA biogenesis in mosquitoes." Cell Rep. **42**(3): 112257.

Riabinina, O., M. Quinn and J. P. Whitehead (2022). "Genetic Toolbox Approaches in Mosquitoes." Cold Spring Harb. Protoc. **2022**(11): Pdb.top107691.

Rivas, F. V., N. H. Tolia, J.-J. Song, J. P. Aragon, J. Liu, G. J. Hannon and L. Joshua-Tor (2005). "Purified Argonaute2 and an siRNA form recombinant human RISC." Nat. Struct. Mol. Biol. **12**(4): 340-349.

Robine, N., N. C. Lau, S. Balla, Z. Jin, K. Okamura, S. Kuramochi-Miyagawa, M. D. Blower and E. C. Lai (2009). "A Broadly Conserved Pathway Generates 3′UTR-Directed Primary piRNAs." Curr. Biol. **19**(24): 2066-2076.

Robinson, A. S. (2005). "Genetic basis of the sterile insect technique." Sterile insect technique: principles and practice in area.

Rose, N. H., A. Badolo, M. Sylla, J. Akorli, S. Otoo, A. Gloria-Soria, J. R. Powell, B. J. White, J. E. Crawford and C. S. McBride (2023). "Dating the origin and spread of specialization on human hosts in Aedes aegypti mosquitoes." Elife **12**.

Rose, N. H., M. Sylla, A. Badolo, J. Lutomiah, D. Ayala, O. B. Aribodor, N. Ibe, J. Akorli, S. Otoo, J.-P. Mutebi, A. L. Kriete, E. G. Ewing, R. Sang, A. Gloria-Soria, J. R. Powell, R. E. Baker, B. J. White, J. E. Crawford and C. S. McBride (2020). "Climate and Urbanization Drive Mosquito Preference for Humans." Curr. Biol. **30**(18): 3570-3579.e3576.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault and M. Solignac (1997). "Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods." Proceedings of the Royal Society of London. Series B: Biological Sciences **250**(1328): 91-98.

Ryan, P. A., A. P. Turley, G. Wilson, T. P. Hurst, K. Retzki, J. Brown-Kenyon, L. Hodgson, N. Kenny, H. Cook, B. L. Montgomery, C. J. Paton, S. A. Ritchie, A. A. Hoffmann, N. P. Jewell, S. K. Tanamas, K. L. Anders, C. P. Simmons and S. L. O'Neill (2019). "Establishment of wMel Wolbachia in Aedes aegypti mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia." Gates Open Res **3**: 1547.

Ryan, S. J., C. J. Carlson, E. A. Mordecai and L. R. Johnson (2019). "Global expansion and redistribution of Aedes-borne virus transmission risk with climate change." PLoS Negl. Trop. Dis. **13**(3): e0007213.

Saito, K., S. Inagaki, T. Mituyama, Y. Kawamura, Y. Ono, E. Sakota, H. Kotani, K. Asai, H. Siomi and M. C. Siomi (2009). "A regulatory circuit for piwi by the large Maf gene traffic jam in Drosophila." Nature **461**(7268): 1296-1299.

Saito, K., H. Ishizu, M. Komai, H. Kotani, Y. Kawamura, K. M. Nishida, H. Siomi and M. C. Siomi (2010). "Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in Drosophila." Genes Dev. **24**(22): 2493-2498.

Saito, K., K. M. Nishida, T. Mori, Y. Kawamura, K. Miyoshi, T. Nagami, H. Siomi and M. C. Siomi (2006). "Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome." Genes Dev. **20**(16): 2214-2222.

Saito, K., Y. Sakaguchi, T. Suzuki, T. Suzuki, H. Siomi and M. C. Siomi (2007). "Pimet, the Drosophila homolog of HEN1, mediates 2'-O-methylation of Piwi- interacting RNAs at their 3' ends." Genes Dev. **21**(13): 1603-1608.

Salazar, M. I., J. H. Richardson, I. Sánchez-Vargas, K. E. Olson and B. J. Beaty (2007). "Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes." BMC Microbiol. **7**: 9.

Saldaña, M. A., K. Etebari, C. E. Hart, S. G. Widen, T. G. Wood, S. Thangamani, S. Asgari and G. L. Hughes (2017). "Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes." PLoS Negl. Trop. Dis. **11**(7): e0005760.

Samuel, G. H., Z. N. Adelman and K. M. Myles (2018). "Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes." Trends Microbiol. **26**(5): 447-461.

Samuel, G. H., T. Pohlenz, Y. Dong, N. Coskun, Z. N. Adelman, G. Dimopoulos and K. M. Myles (2023). "RNA interference is essential to modulating the pathogenesis of mosquito-borne viruses in the yellow fever mosquito Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **120**(11): e2213701120.

Sánchez-Vargas, I., J. C. Scott, B. K. Poole-Smith, A. W. E. Franz, V. Barbosa-Solomieu, J. Wilusz, K. E. Olson and C. D. Blair (2009). "Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway." PLoS Pathog. **5**(2): e1000299.

Schairer, C. E., C. Triplett, O. S. Akbari and C. S. Bloss (2022). "California Residents' Perceptions of Gene Drive Systems to Control Mosquito-Borne Disease." Front Bioeng Biotechnol **10**: 848707.

Schmid-Burgk, J. L., K. Höning, T. S. Ebert and V. Hornung (2016). "CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism." Nat. Commun. **7**: 12338.

Schmidt, T. L., N. H. Barton, G. Rašić, A. P. Turley, B. L. Montgomery, I. Iturbe-Ormaetxe, P. E. Cook, P. A. Ryan, S. A. Ritchie, A. A. Hoffmann, S. L. O'Neill and M. Turelli (2017). "Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti." PLoS Biol. **15**(5): e2001894.

Schnettler, E., C. L. Donald, S. Human, M. Watson, R. W. C. Siu, M. McFarlane, J. K. Fazakerley, A. Kohl and R. Fragkoudis (2013). "Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells." J. Gen. Virol. **94**(Pt 7): 1680-1689.

Schwarz, D. S., Y. Tomari and P. D. Zamore (2004). "The RNA-induced silencing complex is a Mg2+-dependent endonuclease." Curr. Biol. **14**(9): 787-791.

Scott, J. C., D. E. Brackney, C. L. Campbell, V. Bondu-Hawkins, B. Hjelle, G. D. Ebel, K. E. Olson and C. D. Blair (2010). "Comparison of dengue virus type 2-specific small RNAs from RNA interference-competent and -incompetent mosquito cells." PLoS Negl. Trop. Dis. **4**(10): e848.

Scott, T. W., G. G. Clark, L. H. Lorenz, P. H. Amerasinghe, P. Reiter and J. D. Edman (1993). "Detection of multiple blood feeding in Aedes aegypti (Diptera: Culicidae) during a single gonotrophic cycle using a histologic technique." J. Med. Entomol. **30**(1): 94-99.

Scully, R., A. Panday, R. Elango and N. A. Willis (2019). "DNA double-strand break repair-pathway choice in somatic mammalian cells." Nat. Rev. Mol. Cell Biol. **20**(11): 698-714.
Servick, K. (2019). "GM mosquito study draws fire." Science **365**(6459): 1234.

Shanahan, T. M., N. P. McKay, K. A. Hughen, J. T. Overpeck, B. Otto-Bliesner, C. W. Heil, J. King, C. A. Scholz and J. Peck (2015). "The time-transgressive termination of the African Humid Period." Nat. Geosci. **8**(2): 140-144.

Shaw, W. R. and F. Catteruccia (2019). "Vector biology meets disease control: using basic research to fight vector-borne diseases." Nat Microbiol **4**(1): 20-34.

Shi, Y., N. Kopparapu, L. Ohler and D. J. Dickinson (2023). "Efficient and rapid fluorescent protein knock-in with universal donors in mouse embryonic stem cells." Development **150**(10).
Shirai, Y., M. Takahashi, M. Ote, H. Kanuka and T. Daimon (2023). "DIPA-CRISPR gene editing in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae)." Appl. Entomol. Zool. **58**(3): 273-278.

Shousha, A. T. (1948). "Species-eradication: The Eradication of Anopheles gambioe from Upper Egypt, 1942-1945." Bull. World Health Organ. **1**(2): 309-352.

Sienski, G., D. Dönertas and J. Brennecke (2012). "Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression." Cell **151**(5): 964-980.

Sim, S., J. L. Ramirez and G. Dimopoulos (2012). "Dengue virus infection of the Aedes aegypti salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior." PLoS Pathog. **8**(3): e1002631.

Smidler, A. L., O. Terenzi, J. Soichot, E. A. Levashina and E. Marois (2013). "Targeted mutagenesis in the malaria mosquito using TALE nucleases." PLoS One **8**(8): e74511.

Smith, R. H. and R. C. von Borstel (1972). "Genetic control of insect populations." Science **178**(4066): 1164-1174.

Song, J.-J., J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon and L. Joshua-Tor (2003). "The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes." Nat. Struct. Biol. **10**(12): 1026-1032.

Song, J.-J., S. K. Smith, G. J. Hannon and L. Joshua-Tor (2004). "Crystal structure of Argonaute and its implications for RISC slicer activity." Science **305**(5689): 1434-1437.

Souza-Neto, J. A., S. Sim and G. Dimopoulos (2009). "An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense." Proc. Natl. Acad. Sci. U. S. A. **106**(42): 17841-17846.
Spadar, A., J. E. Phelan, E. D. Benavente, M. Campos, L. F. Gomez, F. Mohareb, T. G. Clark and S. Campino (2021). "Flavivirus integrations in Aedes aegypti are limited and highly conserved across samples from different geographic regions unlike integrations in Aedes albopictus." Parasit. Vectors **14**(1): 332.

Sudharshan, S., R. Naidu, M. Mallavarapu and N. Bolan (2012). "DDT remediation in contaminated soils: a review of recent studies." Biodegradation **23**(6): 851-863.

Sun, R., M. Li, C. J. McMeniman and O. S. Akbari (2022). "CRISPR-Mediated Genome Engineering in Aedes aegypti." Methods Mol. Biol. **2509**: 23-51.

Sun, Y. H., L. H. Xie, X. Zhuo, Q. Chen, D. Ghoneim, B. Zhang, J. Jagne, C. Yang and X. Z. Li (2017). "Domestic chickens activate a piRNA defense against avian leukosis virus." Elife **6**.

Suzuki, Y., A. Baidaliuk, P. Miesen, L. Frangeul, A. B. Crist, S. H. Merkling, A. Fontaine, S. Lequime, I. Moltini-Conclois, H. Blanc, R. P. van Rij, L. Lambrechts and M.-C. Saleh (2020). "Non-retroviral endogenous viral element limits cognate virus replication in Aedes aegypti ovaries." Curr. Biol.

Suzuki, Y., L. Frangeul, L. B. Dickson, H. Blanc, Y. Verdier, J. Vinh, L. Lambrechts and M.-C. Saleh (2017). "Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes." J. Virol. **91**(15).

Swarts, D. C., K. Makarova, Y. Wang, K. Nakanishi, R. F. Ketting, E. V. Koonin, D. J. Patel and J. van der Oost (2014). "The evolutionary journey of Argonaute proteins." Nat. Struct. Mol. Biol. **21**(9): 743-753.

Tabachnick, W. J. (1991). "Evolutionary Genetics and Arthropod-borne Disease: The Yellow Fever Mosquito." Am. Entomol. **37**(1): 14-26.

Takken, W., W. B. Snellen, J. P. Verhave, B. G. J. Knols and S. Atmosoedjono (1990). "Environmental measures for malaria control in Indonesia -an historical review on species sanitation." WAGENINGEN AGRICULTURAL UNIVERSITY PAPERS.

Tassetto, M., M. Kunitomi, Z. J. Whitfield, P. T. Dolan, I. Sánchez-Vargas, M. Garcia-Knight, I. Ribiero, T. Chen, K. E. Olson and R. Andino (2019). "Control of RNA viruses in mosquito cells through the acquisition of vDNA and endogenous viral elements." Elife **8**.

Terenius, O., O. Marinotti, D. Sieglaff and A. A. James (2008). "Molecular genetic manipulation of vector mosquitoes." Cell Host Microbe **4**(5): 417-423.

Thizy, D., L. Pare Toe, C. Mbogo, D. Matoke-Muhia, V. P. Alibu, S. K. Barnhill-Dilling, T. Chantler, G. Chongwe, J. Delborne, L. Kapiriri, E. Nassonko Kavuma, S. Koloi-Keaikitse, A. Kormos, K. Littler, D. Lwetoijera, R. Vargas de Moraes, N. Mumba, L. Mutengu, S. Mwichuli, S. E. Nabukenya, J. Nakigudde, P. Ndebele, C. Ngara, E. Ochomo, S. Odiwuor Ondiek, S. Rivera, A. J. Roberts, B. Robinson, R. Sambakunsi, A. Saxena, N. Sykes, B. B. Tarimo, N. Tiffin and K. H. Tountas (2021). "Proceedings of an expert workshop on community agreement for gene drive research in Africa - Co-organised by KEMRI, PAMCA and Target Malaria." Gates Open Res **5**: 19.

Tungu, P., B. Kabula, T. Nkya, P. Machafuko, E. Sambu, B. Batengana, W. Sudi, Y. A. Derua, V. Mwingira, D. Masue, R. Malima, C. Kitojo, N. Serbantez, E. J. Reaves, C. Mwalimu, S. L. Nhiga, M. Ally, H. R. Mkali, J. J. Joseph, A. Chan, J. Ngondi, S. Lalji, S. Nyinondi, E. Eckert, R. Reithinger, S. Magesa and W. N. Kisinza (2023). "Trends of insecticide resistance monitoring in mainland Tanzania, 2004-2020." Malar. J. **22**(1): 100.

Unckless, R. L., A. G. Clark and P. W. Messer (2017). "Evolution of Resistance Against CRISPR/Cas9 Gene Drive." Genetics **205**(2): 827-841.

UniProt, C. (2023). "UniProt: the Universal Protein Knowledgebase in 2023." Nucleic Acids Res. **51**(D1): D523-D531.

Vagin, V. V., A. Sigova, C. Li, H. Seitz, V. Gvozdev and P. D. Zamore (2006). "A distinct small RNA pathway silences selfish genetic elements in the germline." Science **313**(5785): 320-324.

van den Berg, H., H. S. da Silva Bezerra, S. Al-Eryani, E. Chanda, B. N. Nagpal, T. B. Knox, R. Velayudhan and R. S. Yadav (2021). "Recent trends in global insecticide use for disease vector control and potential implications for resistance management." Sci. Rep. **11**(1): 23867.

van Rij, R. P., M.-C. Saleh, B. Berry, C. Foo, A. Houk, C. Antoniewski and R. Andino (2006). "The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster." Genes Dev. **20**(21): 2985-2995.

Varjak, M., C. L. Donald, T. J. Mottram, V. B. Sreenu, A. Merits, K. Maringer, E. Schnettler and A. Kohl (2017). "Characterization of the Zika virus induced small RNA response in Aedes aegypti cells." PLoS Negl. Trop. Dis. **11**(10): e0006010.

Varjak, M., K. Maringer, M. Watson, V. B. Sreenu, A. C. Fredericks, E. Pondeville, C. L. Donald, J. Sterk, J. Kean, M. Vazeille, A.-B. Failloux, A. Kohl and E. Schnettler (2017). "Aedes aegypti Piwi4 Is a Noncanonical PIWI Protein Involved in Antiviral Responses." mSphere **2**(3).

Venkataraman, K., N. Shai, P. Lakhiani, S. Zylka, J. Zhao, M. Herre, J. Zeng, L. A. Neal, H. Molina, L. Zhao and L. B. Vosshall (2023). "Two novel, tightly linked, and rapidly evolving genes underlie Aedes aegypti mosquito reproductive resilience during drought." Elife **12**.

Vodovar, N., A. W. Bronkhorst, K. W. R. van Cleef, P. Miesen, H. Blanc, R. P. van Rij and M.-C. Saleh (2012). "Arbovirus-derived piRNAs exhibit a ping-pong signature in mosquito cells." PLoS One **7**(1): e30861.

Wang, W., B. W. Han, C. Tipping, D. T. Ge, Z. Zhang, Z. Weng and P. D. Zamore (2015). "Slicing and Binding by Ago3 or Aub Trigger Piwi-Bound piRNA Production by Distinct Mechanisms." Mol. Cell **59**(5): 819-830.

Wang, Y., B. Jin, P. Liu, J. Li, X. Chen and J. Gu (2018). "piRNA Profiling of Dengue Virus Type 2-Infected Asian Tiger Mosquito and Midgut Tissues." Viruses **10**(4).

Wang, Y., S. Juranek, H. Li, G. Sheng, T. Tuschl and D. J. Patel (2008). "Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex." Nature **456**(7224): 921-926.

Weaver, S. C. and W. K. Reisen (2010). "Present and future arboviral threats." Antiviral Res. **85**(2): 328-345.

Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed and J. J. Welch (2015). "The incidence of bacterial endosymbionts in terrestrial arthropods." Proc. Biol. Sci. **282**(1807): 20150249.

Werren, J. H., L. Baldo and M. E. Clark (2008). "Wolbachia: master manipulators of invertebrate biology." Nat. Rev. Microbiol. **6**(10): 741-751.

Whitfield, Z. J., P. T. Dolan, M. Kunitomi, M. Tassetto, M. G. Seetin, S. Oh, C. Heiner, E. Paxinos and R. Andino (2017). "The Diversity, Structure, and Function of Heritable Adaptive Immunity Sequences in the Aedes aegypti Genome." Curr. Biol. **27**(22): 3511-3519.e3517.

Who (2023). "Geographical expansion of cases of dengue and chikungunya beyond the historical areas of transmission in the Region of the Americas."

Wilson, A. L., O. Courtenay, L. A. Kelly-Hope, T. W. Scott, W. Takken, S. J. Torr and S. W. Lindsay (2020). "The importance of vector control for the control and elimination of vector-borne diseases." PLoS Negl. Trop. Dis. **14**(1): e0007831.

Wu, H., R. Pang, T. Cheng, L. Xue, H. Zeng, T. Lei, M. Chen, S. Wu, Y. Ding, J. Zhang, M. Shi and Q. Wu (2020). "Abundant and Diverse RNA Viruses in Insects Revealed by RNA-Seq Analysis: Ecological and Evolutionary Implications." mSystems **5**(4).

Wu, P.-H., Y. Fu, K. Cecchini, D. M. Özata, A. Arif, T. Yu, C. Colpan, I. Gainetdinov, Z. Weng and P. D. Zamore (2020). "The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility." Nat. Genet. **52**(7): 728-739.

Xi, Z., J. L. Ramirez and G. Dimopoulos (2008). "The Aedes aegypti toll pathway controls dengue virus infection." PLoS Pathog. **4**(7): e1000098.

Xiao, X., Y. Liu, X. Zhang, J. Wang, Z. Li, X. Pang, P. Wang and G. Cheng (2014). "Complement-related proteins control the flavivirus infection of Aedes aegypti by inducing antimicrobial peptides." PLoS Pathog. **10**(4): e1004027.

Xiol, J., P. Spinelli, M. A. Laussmann, D. Homolka, Z. Yang, E. Cora, Y. Couté, S. Conn, J. Kadlec, R. Sachidanandam, M. Kaksonen, S. Cusack, A. Ephrussi and R. S. Pillai (2014). "RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts." Cell **157**(7): 1698-1711.

Yadav, A. K., C. Butler, A. Yamamoto, A. A. Patil, A. L. Lloyd and M. J. Scott (2023). "CRISPR/Cas9-based split homing gene drive targeting doublesex for population suppression of the global fruit pest Drosophila suzukii." Proc. Natl. Acad. Sci. U. S. A. **120**(25): e2301525120.

Yamaguchi, S., A. Oe, K. M. Nishida, K. Yamashita, A. Kajiya, S. Hirano, N. Matsumoto, N. Dohmae, R. Ishitani, K. Saito, H. Siomi, H. Nishimasu, M. C. Siomi and O. Nureki (2020). "Crystal structure of Drosophila Piwi." Nat. Commun. **11**(1): 858.

Yang, W. and T. A. Steitz (1995). "Recombining the structures of HIV integrase, RuvC and RNase H." Structure **3**(2): 131-134.

Yao, F. A., A.-A. Millogo, P. S. Epopa, A. North, F. Noulin, K. Dao, M. Drabo, C. Guissou, S. Kekele, M. Namountougou, R. K. Ouedraogo, L. Pare, N. Barry, R. Sanou, H. Wandaogo, R. K. Dabire, A. McKemey, F. Tripet and A. Diabaté (2022). "Mark-release-recapture experiment in Burkina Faso demonstrates reduced fitness and dispersal of genetically-modified sterile malaria mosquitoes." Nat. Commun. **13**(1): 796.

Yen, J. H. and A. R. Barr (1973). "The etiological agent of cytoplasmic incompatibility in Culex pipiens." J. Invertebr. Pathol. **22**(2): 242-250.

Yuan, Y.-R., Y. Pei, J.-B. Ma, V. Kuryavyi, M. Zhadina, G. Meister, H.-Y. Chen, Z. Dauter, T. Tuschl and D. J. Patel (2005). "Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage." Mol. Cell **19**(3): 405-419.

Zhang, F., J. Wang, J. Xu, Z. Zhang, B. S. Koppetsch, N. Schultz, T. Vreven, C. Meignin, I. Davis, P. D. Zamore, Z. Weng and W. E. Theurkauf (2012). "UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery." Cell **151**(4): 871-884.

Zheng, K. and P. J. Wang (2012). "Blockade of pachytene piRNA biogenesis reveals a novel requirement for maintaining post-meiotic germline genome integrity." PLoS Genet. **8**(11): e1003038.

Zheng, X., D. Zhang, Y. Li, C. Yang, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, J. Bouyer, M. Tang, B. Zheng, J. Yu, J. Liu, J. Zhuang, Z. Hu, M. Zhang, J.-T. Gong, X.-Y. Hong, Z. Zhang, L. Lin, Q. Liu, Z. Hu, Z. Wu, L. A. Baton, A. A. Hoffmann and Z. Xi (2019). "Incompatible and sterile insect techniques combined eliminate mosquitoes." Nature **572**(7767): 56-61.

Zhu, G.-H., N. M. Albishi, X. Chen, R. L. Brown and S. R. Palli (2021). "Expanding the Toolkit for Genome Editing in a Disease Vector, Aedes aegypti: Transgenic Lines Expressing Cas9 and Single Guide RNA Induce Efficient Mutagenesis." CRISPR J **4**(6): 846-853.

## TABLES

| NAME | DNA SEQUENCE | LOCATION |
|---|---|---|
| Ago3 gRNA1 | CAACGTAAAGGAATTGGACG | 2:272560073 - 272560054 |
| Ago3 gRNA3 | TCGGAGACAACCGATGGCGC | 2:272559871-272559890 |
| Piwi2 gRNA1 | GACACTGTATCAGTATCGTG | 1:62490523-62490542 |
| Piwi2 gRNA2 | CAGAACAACGACTCCCAGGA | 1:62491044-62491063 |
| Piwi4 gRNA1 | CAGCTGGAGAGCCTGACCCG | 2:470367316-470367297 |
| Piwi4 gRNA2 | GGAGCGTATCTCCAACGCCA | 2:470367257-470367238 |
| Piwi5 gRNA1 | TGGTGTCTCGTGCTAAGCAG | 3:279945889-279945870 |
| Piwi5 gRNA2 | AGTAGATGGCACGAATGAGA | 3:279946368-279946349 |
| Piwi6 gRNA1 | TCTCGGGGTGGCTACAACGG | 3:279915320-279915301 |
| Piwi6 gRNA2 | GGGGGATCCTCATCTCGGGG | 3:279915332-279915313 |
| Ae White gRNA1 | GTCCTTCAGGTCGCCTC | 1:107955475 - 107955456 |
| Ae White gRNA2 | CAGGGTTTCGGATGCAA | 1:107955133 - 107955152 |
| Ae Ebony-1 gRNA | CGTGTTCGGCGCAACGC | 1:201181504 - 201181523 |
| Ae Ebony-2 gRNA | CGTTGCAGATGACAGCCG | 1:201181193 - 201181212 |
| Ae Ebony-3 gRNA | GACGGCCAACCGCGTGGCGG | 1:201181249 - 201181271 |
| Ae Ebony-4 gRNA | ATGTAATCTCCGTCCCGATT | 1:201181332 - 201181313 |

**Table 2.1:** A list of PIWI sgRNAs used in this chapter. The location of each sgRNA is indicated according to the AaegL5.3 *Ae. aegypti* genome build.

| Primer name | Primer sequence |
|---|---|
| Ago3 gRNA 1/2 For | GGAGTTTCTAAACGTACGACGG |
| Ago3 gRNA3 Rev | CTTCGGAGAGTTCGTCATGTTT |
| Ago3 gRNA3 Rev2 | GCTCGATCCAGTCTTGATGA |
| Piwi2 gRNA1 For3 | GGACAAGCCGTGGAGCGAT |
| Piwi2 gRNA2 Rev3 | GGCCATCAGTTCGTTACCAC |
| Piwi4 gRNA For2 | CGATCGAGGGAACCGAGAGA |
| Piwi4 gRNA2 Rev3 | GACCATGAACAGTTGAGTTCCG |
| Piwi5 gRNA1 For2 | TCTCGTGTCCATCAATCTTG |
| Piwi5 gRNA1 Rev2 | TCTGAAGGGCACCATTGGT |
| Piwi6 gRNA1 For2 | ATAATTGGCCGTCCGGAGTT |
| Piwi6 gRNA2 Rev2 | GCCTACAATCAGTGCTCGGTTA |
| White For | TAAGAGGCAAGACACCACAAG |
| White Rev | GCACGAACGACTCACATACA |

**Table 2.2:** A list of the primers used to amplify the *piwi* genes around the sgRNA target site.

| Gene target | Distance between sgRNAs | Distance between F primer & sgRNA1 | Distance between R primer & sgRNA2 | PCR amplicon size |
|---|---|---|---|---|
| Ago3 | 165 bp | 185 bp | 143 bp | 531 bp |
| Piwi2 | 507 bp | 165 bp | 227 bp | 899 bp |
| Piwi4 | 60 bp | 314 bp | 185 bp | 558 bp |
| Piwi5 | 479 bp | 122 bp | 144 bp | 768 bp |
| Piwi6 | 12 bp | 303 bp | 540 bp | 887 bp |

**Table 2.3:** PIWI PCR amplicon sizes and the distances between the potential cut sites. The distances between the F primer and sgRNA1, and the R primer and sgRNA2 give information on the potential PCR band sizes depending on which sgRNA is effective, allowing for limited diagnosis of editing events from PCR amplicons run on higher percentage agarose gels.

| Target | Distance between sgRNAs | No. eggs injected | Larvae | Hatch frequency (%) | Pupae | Adults | Eclosion frequency (%) | Survival frequency (%) | Positive T7E1 | Positive sequencing | Phenotype |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *White* | 300 bp | 108 | 44 | 40.7 | 28 | 23 | 52.3 | 21.3 | 9 (8.3%) | 4 (3.7%) | N/A |
| *Ago3* | 164 bp | 262 | 30 | 11.5 | 15 | 15 | 50 | 5.7 | 1L; 1M (0.8%) | 1L; 1M (0.8%) | Unusual death at larval stage |
| *Piwi2* | 507 bp | 328 | 40 | 12.2 | 36 | 25 | 62.5 | 7.6 | 1M (0.3%) | 1M (0.3%) | Unusual pupal death; early adult female death |
| *Piwi4* | 60 bp | 203 | 36 | 17.7 | 35 | 30 | 83.3 | 14.8 | 4M; 2F (2.96%) | 2M (0.99%) | Slower larval growth rate |
| *Piwi5* | 479 bp | 217 | 20 | 9.2 | 18 | 17 | 85 | 7.8 | 2M; 3F (2.3%) | 1M (0.5%) | Adults may have matured slowly |
| *Piwi6* | 10 bp | 230 | 32 | 13.9 | 32 | 30 | 93.8 | 13.04 | 3M; 2F (2.2%) | 3M; 2F (2.2%) | None |

**Table 2.4:** Results of the *piwi* knock-out NHEJ experiment. Two sgRNAs per target gene were injected into the embryos and the number of animals at each life stage was recorded. The presence of indels was inferred using the T7 Endonuclease I assay. Positive T7 assays were confirmed by Sanger sequencing of the targeted regions.

| Target | Eggs injected | Larvae | Hatch frequency (%) | Pupae | Adults | Ecclosion frequency (%) | Survival frequency (%) | Survival from hatched larvae (%) | PAX3-positive | T7 Positive | Confirmed insertion |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ago3 | 793 | 38 | 4.8 | 36 | 33 | 86.8 | 4.16 | 95.12 | — | — | — |
| Piwi2 | 728 | 111 | 15.2 | 103 | 70 | 63.1 | 9.62 | 93.13 | — | — | — |
| Piwi4 | 731 | 53 | 7.3 | 51 | 32 | 60.4 | 4.38 | 92.45 | 3 (5.7%) | 2 (3.8%) | — |
| Piwi5 | 651 | 114 | 17.5 | 112 | 103 | 90.4 | 15.82 | 73.46 | 2 (1.8%) | 2 (1.8%) | 2 (1.8%) |
| Piwi6 | 574 | 24 | 4.2 | 21 | 17 | 70.8 | 2.96 | 70.83 | 2 (8.3%) | 4 (16.7%) | 3 (12.5%) |
| White | 666 | 98 | 14.7 | 92 | 87 | 89.8 | 13.06 | 94.90 | 1 (G1) | — | — |

**Table 2.5:** Results of the first set of CRISPaint knock-in injections. CRISPaint-GFP or CRISPaint-RFP plasmids were added to the injection mix to perform HDR-less insertion of the plasmid at the target site. The G0 pupae were screened for Pax3/3xp3 driven expression of fluorescence in the eye. Insertion of the plasmid was confirmed by amplification across the gene-plasmid junctions, followed by sequencing of PCR clones.

| Target | Eggs injected | Larvae | Hatch frequency (%) | Pupae | Adults | Eclosion frequency (%) | Survival frequency (%) | Survival from hatched larvae (%) | GFP-positive | PCR positive for indels | Confirmed indels |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ago3 | 215 | 55 | 25.58 | 50 | 43 | 86.00 | 20.00 | 78.18 | - | 4 (7.3%) | 1 (1.8%) |
| Piwi2 | 221 | 39 | 17.65 | 39 | 38 | 97.44 | 17.19 | 97.44 | 1 (2.6%) | 2 (5.1%) | 1 (2.6%) |
| Piwi4 | 219 | 23 | 10.50 | 21 | 20 | 95.24 | 9.13 | 86.96 | - | - | - |
| Piwi5 | 215 | 30 | 13.95 | 21 | 16 | 76.19 | 7.44 | 53.33 | - | 3 (10%) | - |
| Piwi6 | 211 | 22 | 10.43 | 22 | 21 | 95.45 | 9.95 | 95.45 | 1 (4.5%) | 1 (4.5%) | 1 (4.5%) |
| Ebony | 226 | 45 | 19.91 | 39 | 33 | 84.62 | 14.60 | 73.33 | 1 (2.2%) | - | - |
| Control | 215 | 82 | 38.14 | 75 | 72 | 96.00 | 33.49 | 87.80 | - | - | - |

**Table 2.6:** Results of the CRISPaint II experiment. Amplification of the target site, followed by electrophoresis on a 1.5% gel was performed to identify potential large deletions which were referred to as PCR positive for indels. Sanger sequencing of the PCR amplicon and analysis of the data using ICE (https://ice.synthego.com/) confirmed the presence of indels in this experiment.

| Ago3 replicate | No. of eggs injected | No. hatched | Hatch frequency (%) | Pupae | GFP+ |
|---|---|---|---|---|---|
| A3-1 | 50 | 7 | 14 | 3 | - |
| A3-2 | 56 | 9 | 16.1 | 5 | - |
| A3-3 | 58 | 12 | 20.7 | 8 | - |
| A3-4 | 60 | 23 | 38.3 | 8 | - |

**Table 2.7:** Repeated Ago3 CRISPaint injections. The injections were repeated to replicate and confirm the relatively high hatch frequencies seen in the CRISPaint II injections. In this experiment, the hatch frequency was 22.8%, validating the previous hatch frequency result.

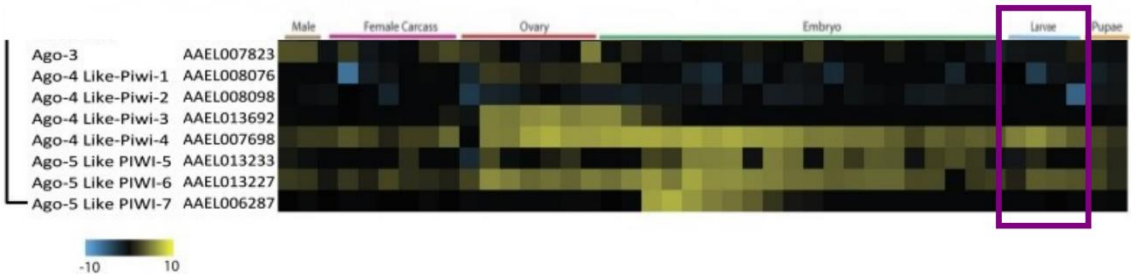


**Figure 2.1**: Heatmap showing the Piwi-subfamily expression in the male, female carcass without ovaries, ovaries at different time points before and after blood-feeding, the embryo at different time points during development, the four larval stages of development and the pupal stage. Image adapted from (Akbari, Antoshechkin et al. 2013).

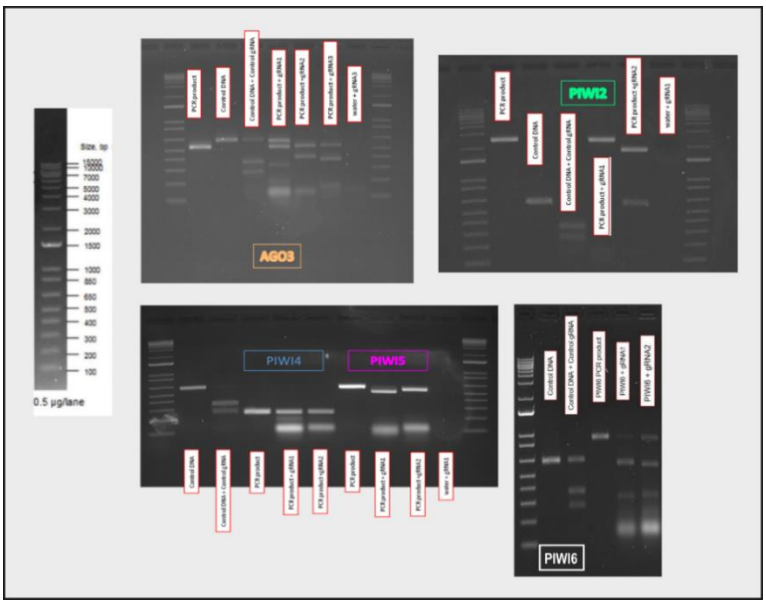


**Figure 2.2:** *in-vitro* cleavage analysis results for piwi sgRNAs used in Chapter 2.

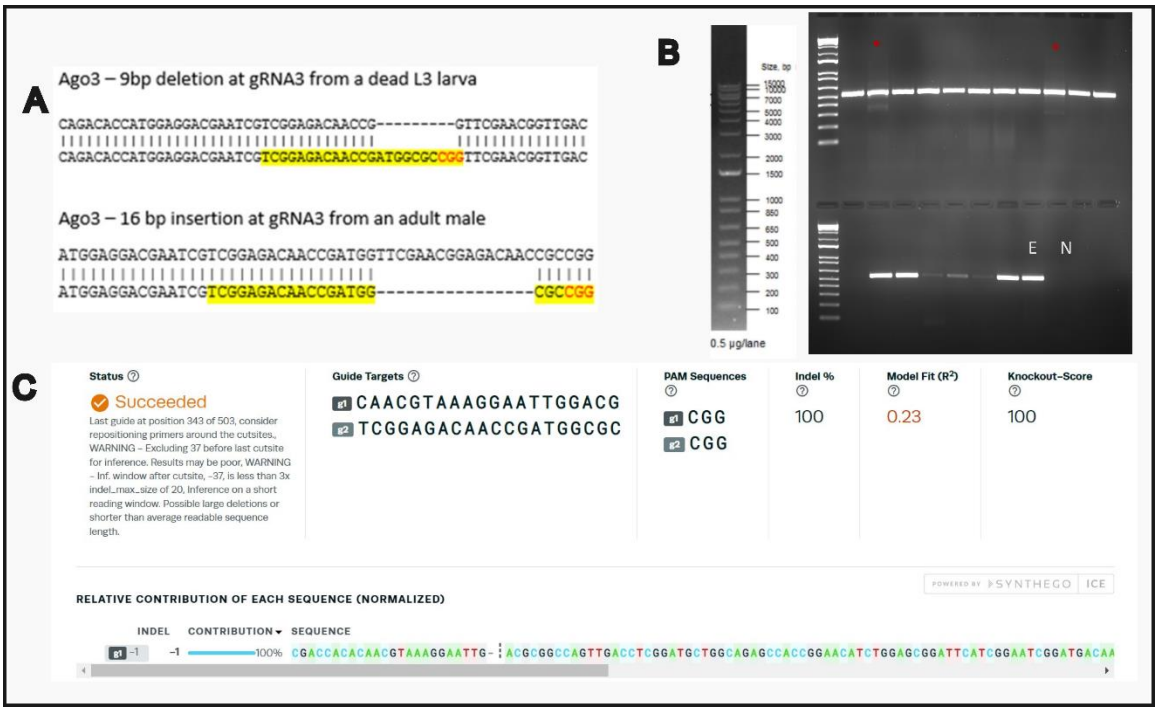


**Figure 2.3:** Examples of successful Ago3 G0 editing results. **3A:** Alleles with indels at the Ago3 sgRNA3 target site. The 9 bp deletion was recovered from sequencing of a T7E1 positive larva that died during development, while the 16 bp insertion was recovered from an adult male that died during soon after eclosing. Only sgRNA3 mediated editing events were recovered throughout all Ago3 injection experiments. **3B:** Electrophoresis of Ago3 PCR amplicons from CRISPaint experiment II on a 1.5% agarose gel. The red stars represent individual G0 mosquitoes that were PCR positive for indels, determined by the presence of a smaller band at approximately 400 bp. This represents possible cleavage at sgRNA3 which would result in a 388 bp band. E – Exu-Cas9 gDNA serves as a positive control, N – negative control. **3C:** Example of positive ICE results from Ago3 G0 CRISPaint II mosquitoes.

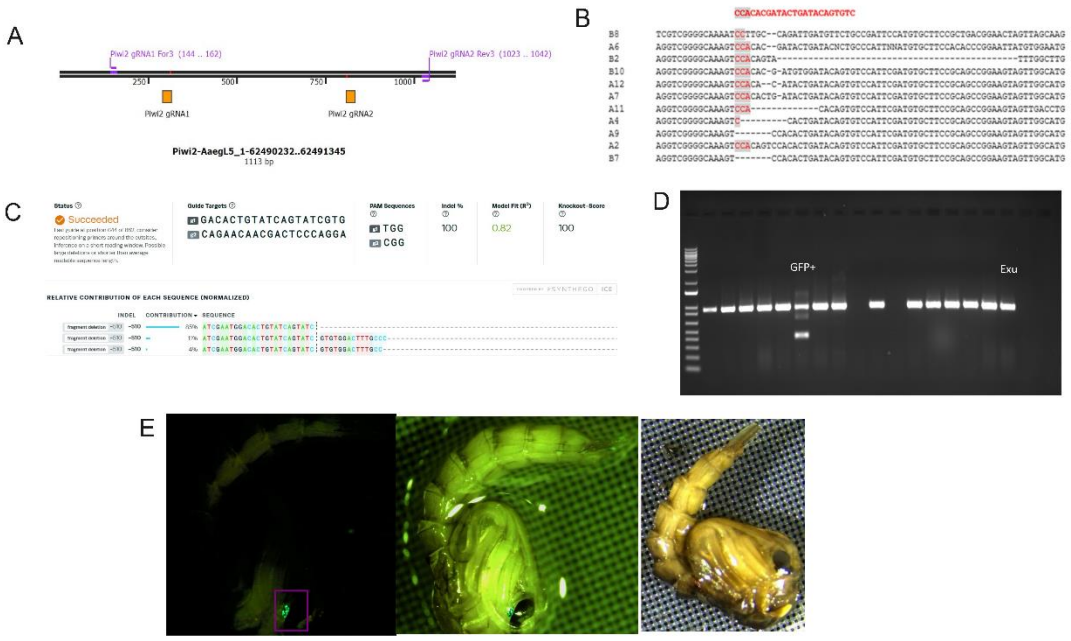


**Figure 2.4:** Piwi2 editing results. **4A:** A schematic of the Piwi2 genomic locus. **4B:** Example of editing at sgRNA1. The mutant alleles were recovered from a G0 male. **4C:** ICE analysis results from sequencing of the pupa shown in 3E. The knockout score was 100% with 3 different large 510 bp deletion alleles. **4D:** PCR analysis of Piwi2 G0 mosquitoes. The GFP+ refers to the pupa in 3E, Exu – background strain Exu-Cas9. **4E:** Piwi2-GFP+ pupa. The left panel shows the pupa under the GFP filter with fluorescence in the eye, the panel in the middle is the same as on the left with the brightfield, while the panel on the right is an example of an Exu-Cas9 pupa.

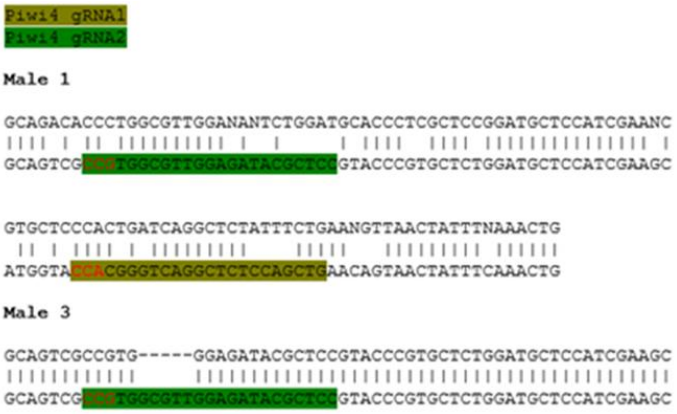


**Figure 2.5:** Example of editing at both Piwi4 sgRNAs. The alleles were recovered from sequencing of T7E1 positive mosquitoes from the NHEJ experiment.

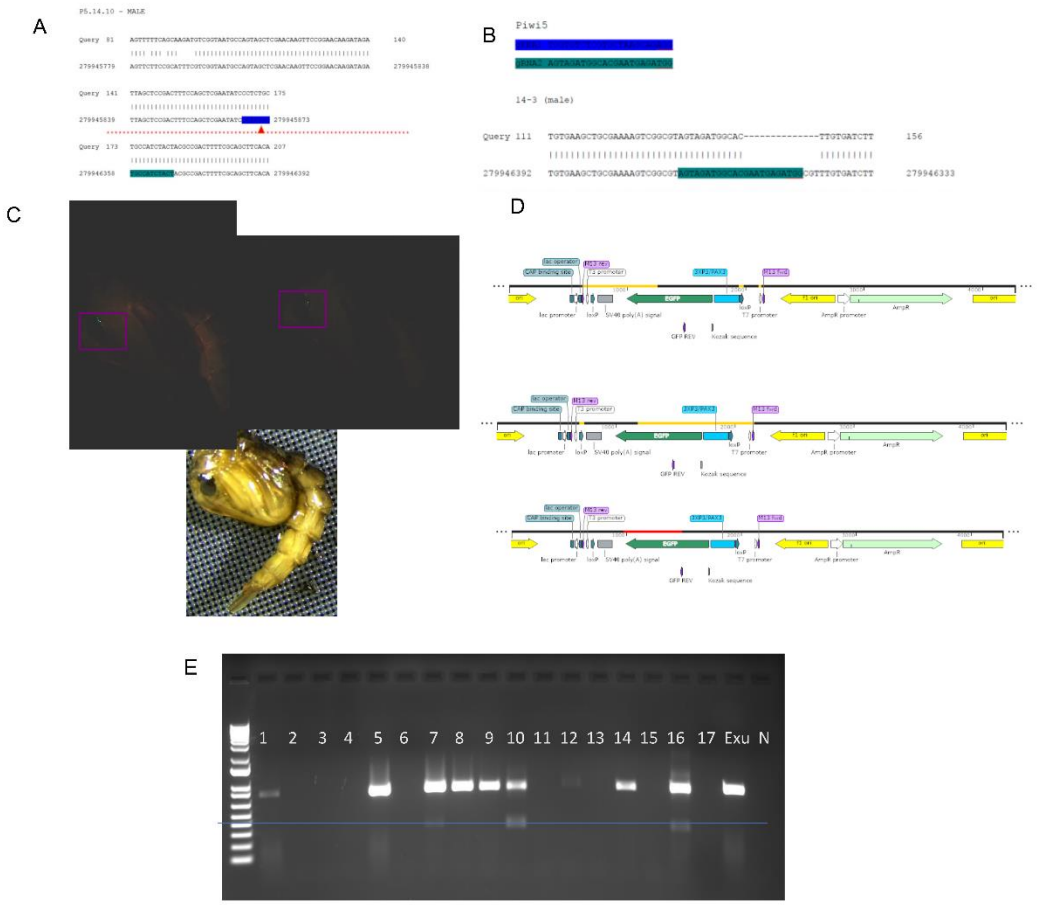


**Figure 2.6:** Results of Piwi5 injections. **6A** and **6B:** alleles recovered from T7E1 positive G0 mosquitoes in the NHEJ experiment. **6C**: Two GFP+ pupae (right – male, left – female) were obtained from the CRISPaint I experiment, shown side-by-side with the background strain, Exu-Cas9 below for comparison. **6D**: Junction PCR across the gene-plasmid junction followed by Sanger sequencing of cloned PCR amplicons resulted in the recovery of the regions of the plasmid highlighted in yellow in the GFP+ female and in red in the GFP+ male. **6E**: PCR analysis of the CRISPaint II experiment. The blue horizontal line represents the size of the amplicon expected in edited G0 individuals.

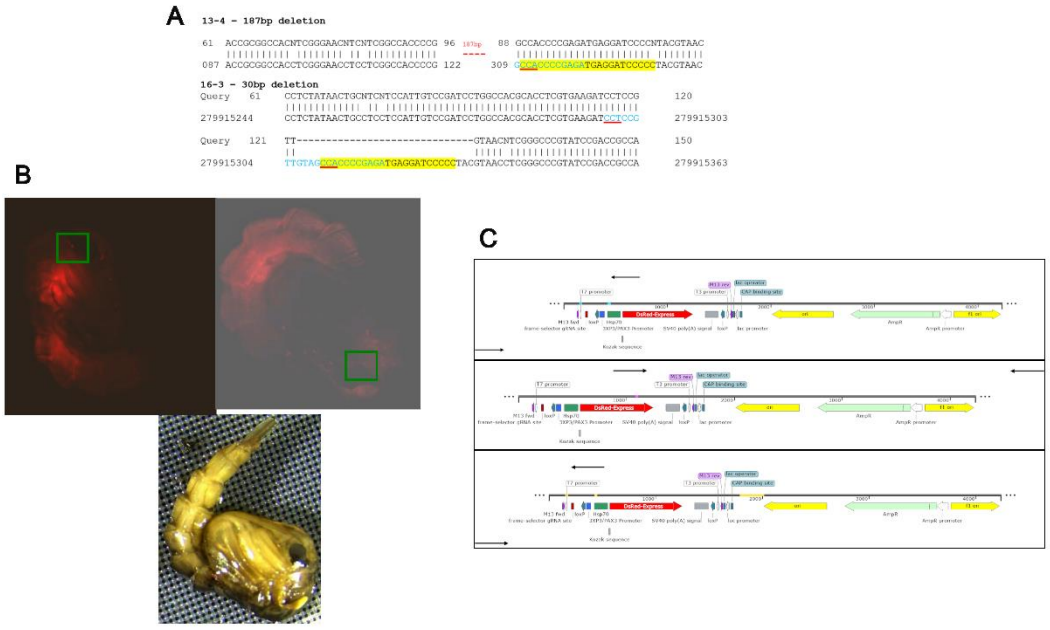


**Figure 2.7:** Piwi6 editing results. **7A:** NHEJ editing results. 5 mosquitoes tested positive in the T7E1 assay and the presence of indels was confirmed by Sanger sequencing PCR clones in all 5 mosquitoes. **7B**: two pupae were positive for RFP fluorescence in the eye. Only the pupa on the left had sequence confirmation of insertion of the plasmid. The background strain, Exu-Cas9 in included for comparison. **7C**: schematic showing regions recovered by sequencing of junction PCR clones highlighted on the plasmid. At least three insertion alleles were recovered, with the amplified region represented by arrows.


**Figure 2.8:** White-GFP+ G1 pupa. None of the G0 mosquitoes were positive for GFP fluorescence in the eye. Screening of G1 pupae led to the discovery of the GFP+ pupa, providing evidence of inheritance of the CRISPaint-GFP plasmid. **8A** shows the pupa under bright field, **8B** shows the pupa under the GFP filter and **8C** is a merge of 8A and 8B.

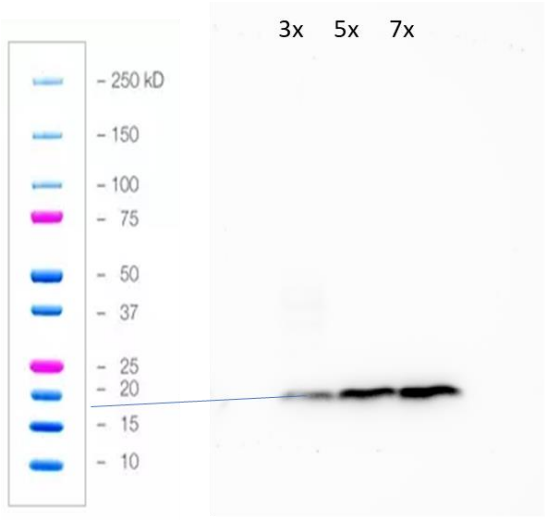


**Figure 2.9:** Piwi4 Western blot with protease and phosphatase inhibitors at different concentrations. the Piwi4 antibody was used at 1:250 dilution, and the inhibitor concentrations tested were 3x, 5x and 7x. Full length protein was not recovered.

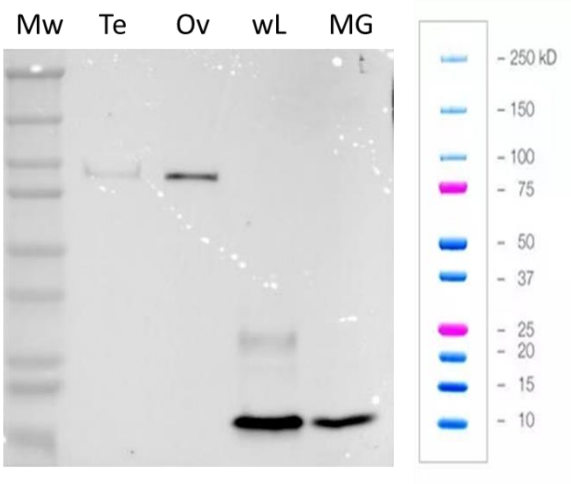


**Figure 2.10:** Piwi4 Western blot results. Mw – molecular weight marker, Te – testes, Ov – ovaries, wL – whole larvae, MG – larval midgut.

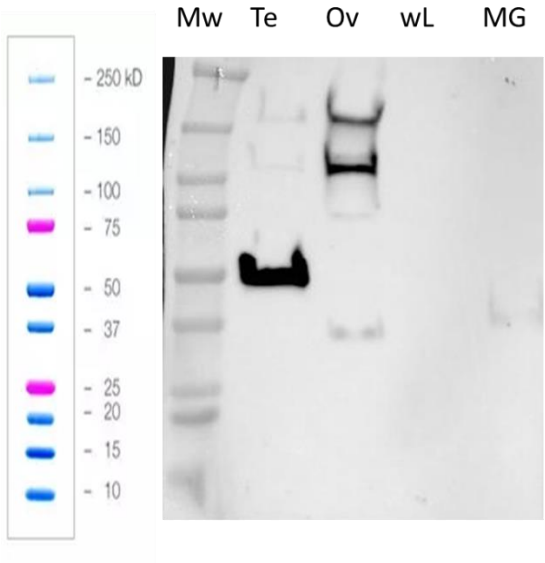


**Figure 2.11:** Piwi5 Western blot (1:250). Mw – molecular weight marker, Te – testes, Ov – ovaries, wL – whole larvae, MG – larval midgut. Full length protein was present in testes, albeit less abundant than in ovaries. Nothing appears in the whole larvae sample, while a shorter band is visible in the midgut sample.

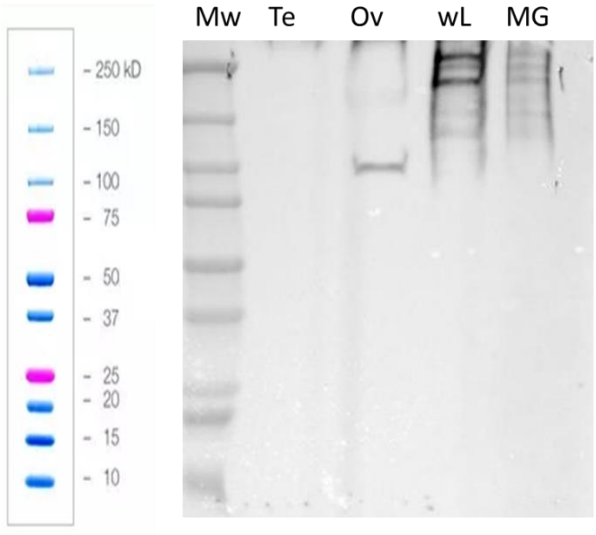


**Figure 2.12:** Piwi6 Western blot (1:250). Mw – molecular weight marker, Te – testes, Ov – ovaries, wL – whole larvae, MG – larval midgut. Piwi6 is only present in the ovaries.

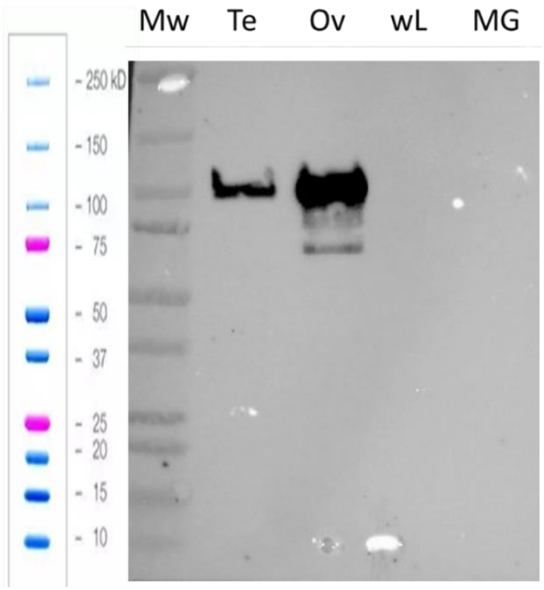


**Figure 2.13:** Piwi2 Western blot results (1:250). Mw – molecular weight marker, Te – testes, Ov – ovaries, wL – whole larvae, MG – larval midgut. Piwi2 was only detected in the testes and blood-fed ovaries.

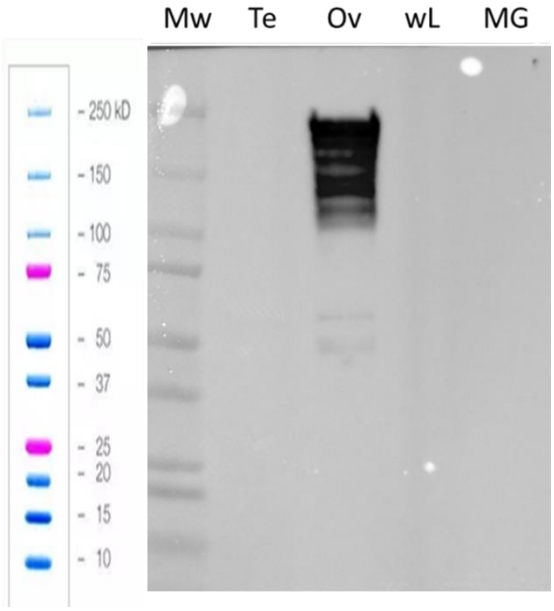


**Figure 2.14**: Ago3 Western blot results (1:250). Mw – molecular weight marker, Te – testes, Ov – ovaries, wL – whole larvae, MG – larval midgut. Ago3 was detected in the blood-fed ovaries as part of a large complex.

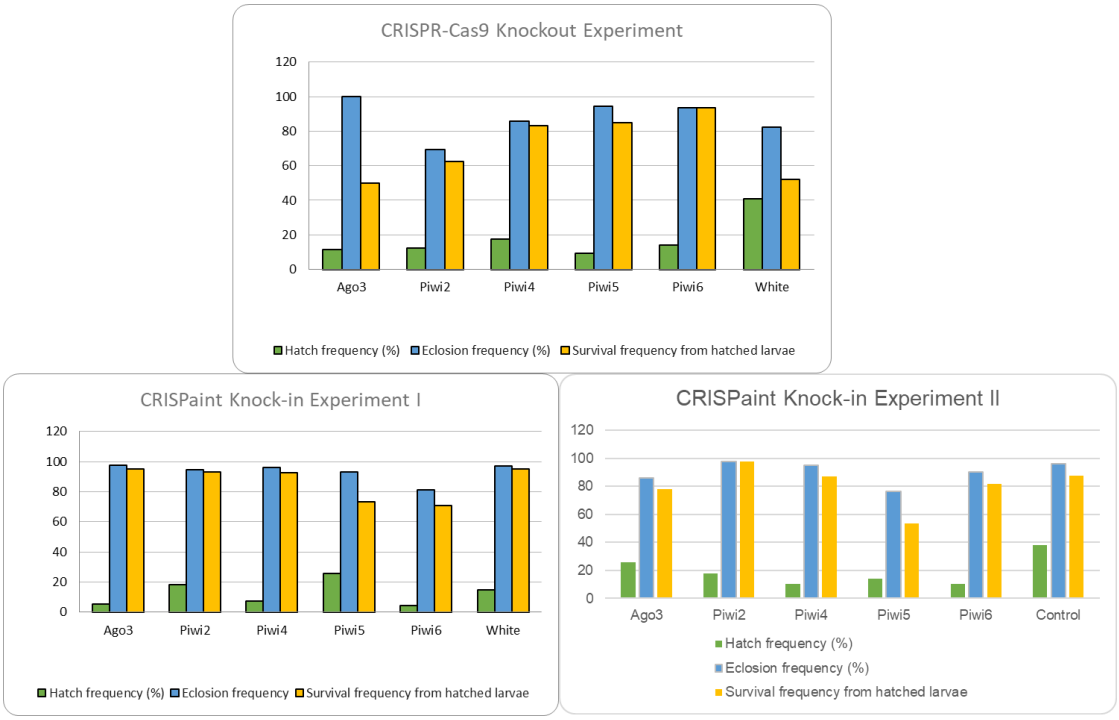


**Figure 2.15:** Graphical representation of the comparison between the hatching, eclosion and survival frequency of the three piwi experiments.

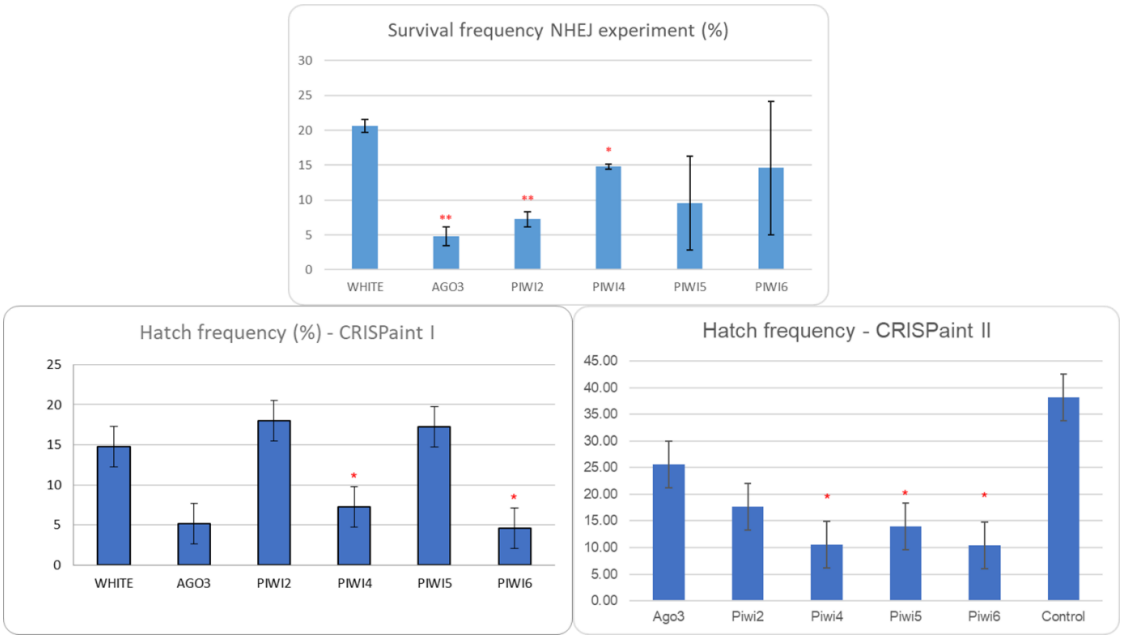


**Figure 2.16:** Comparison of the hatching frequencies between the three *piwi* experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ as calculated by the two-tailed student's T-test.

# CHAPTER THREE

## piRNA ENGINEERING EXPERIMENT

### INTRODUCTION

#### The mosquito *Aedes aegypti* is an efficient vector of arboviruses.

***Aedes aegypti*** **is a major vector of arthropod-borne (arboviruses). This capacity as a successful vector is driven by the ability to maintain a significant viral load without substantial negative effects to the host (Lambrechts and Scott Thomas 2009). The importance of this immune balancing act to the mosquito is underscored by the discovery that most arboviruses are RNA viruses with the main viral genera contributing to the global aedine mosquito-borne disease morbidity and mortality. These include flaviviruses, alphaviruses and phleboviruses, all of which are single-stranded RNA viruses, with the only known DNA arbovirus being African swine fever virus (Weaver and Reisen 2010). Insect-specific viruses (ISVs) are more diverse than arboviruses, however, most ISVs are RNA viruses with flaviviruses being the most represented viral family (de Almeida, Aguiar et al. 2021).**

**The vectorial capacity of *Ae. aegypti* for arboviruses such as Dengue virus (DENV) is projected to increase toward the end of this century, as it is influenced by both temperature and diurnal temperature range which are both projected to provide favorable conditions for active transmission outside of tropical and sub-tropical regions. (Liu-Helmersson, Stenlund et al. 2014).**

## *Ae. aegypti* immunity to viral infection

Insects do not encode antibodies and are said to not have a canonical adaptive immune system, instead relying on an innate immune system to respond to viral infections (Merkling and van Rij 2013). While most data on insect immune responses have been characterized in *D. melanogaster*, we can trace the path of arboviral infection in the mosquito. For a virus acquired through ingestion in either of the feeding stages, i.e., larval and adult, the first barrier to infection is known as the midgut entry barrier, where the virus must infect and replicate efficiently in the midgut epithelial cells, followed by the midgut escape barrier, from where the virus is disseminated to other tissues (Myles, Pierro et al. 2003, Salazar, Richardson et al. 2007, Franz, Kantor et al. 2015, Danet, Beauclair et al. 2019).

Once the virus is circulating in the hemolymph, the humoral innate immune pathway is activated after recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors, which are activated and result in several signaling cascades which lead to increased expression of effector molecules such as antimicrobial peptides (AMPs) (Xiao, Liu et al. 2014). The proteolytic activity associated with the penetration of the midgut entry and escape barriers also acts as a "danger signal" to activate two signal transduction cascades, namely the Toll and IMD pathways, resulting in the activation of transcription factors that give rise to increased expression of genes related to immune defense, including AMPs (El Chamy, Leclerc et al. 2008, Xi, Ramirez et al. 2008). The main site of synthesis of these

effector molecules is the fat body, from where the soluble peptides are released into the hemolymph for circulation (Lemaitre and Hoffmann 2007).

The evolutionarily conserved JAK-STAT signaling pathway also plays an important role in antiviral defense in the yellow fever mosquito (Souza-Neto, Sim et al. 2009). Vago, a secreted immune factor similar to interferon in mammals, acts as the trigger for the JAK-STAT pathway facilitated by increased expression in response to double-stranded RNA recognition mediated by Dicer2 (Paradkar, Trinidad et al. 2012, Paradkar, Duchemin et al. 2014).

While these pathways are known to reduce the replication of viruses in mosquitoes, they are not sufficient to eliminate viral infections, as these are known to persist throughout the life of the infected mosquito (Goic, Vodovar et al. 2013) The viral titer in the midgut decreases as the infection proceeds, while the virus accumulates in tissues such as the salivary glands, facilitating transmission during the next bloodmeal (Salazar, Richardson et al. 2007). The salivary glands also mount their own immune response to viral infection to keep the viral titers under control (Sim, Ramirez et al. 2012).

microRNAs (miRNAs) have not been traditionally associated with antiviral activity as viral replication typically occurs in the cytoplasm, while Drosha, a key protein in miRNA processing is localized to the nucleus (Kim, Han et al. 2009). However, the advent of deep sequencing has allowed the discovery of virus-associated miRNAs which respond to viral infection, however their role in immunity is unclear (Saldaña, Etebari et al. 2017).

Small interfering RNAs (siRNAs) are the key players in the mosquito response to viral infection where the exogenous siRNA (exo-siRNA) pathway is triggered by the presence of double-stranded RNA (dsRNA) in the cytoplasm, produced as replication intermediates of the infecting virus (Li, Li et al. 2004). These viral dsRNA are processed by Dicer2 into antiviral siRNAs, 21 nucleotide single-stranded RNAs which are loaded with the help of R2D2 into the siRNA-induced silencing complex (siRISC) with Ago2 target the cognate virus in a sequence-specific manner for degradation by the endonuclease activity of Ago2 (Adelman, Sanchez-Vargas et al. 2002, Sánchez-Vargas, Scott et al. 2009, Liu and Paroo 2010, Scott, Brackney et al. 2010).

The viral response to host (mosquito) immune system has been investigated with viral suppressors of RNAi (VSRs) discovered in various arboviruses, for example, a DENV protein has been found to interfere with the siRNA pathway by blocking the activity of Dicer2 (Kakumani, Ponia et al. 2013). Loquacious (Loqs), a dsRNA-binding protein that is vital for RNAi is hijacked by flaviviruses to enhance infection in the mosquito (Besson, Lezcano et al. 2022). Similarly, sub-genomic flavivirus RNA (sfRNA) is produced during flaviviral infection and is important in overcoming the midgut barriers to infection (Göertz, Fros et al. 2016). Viruses accumulate mutations as they replicate throughout the mosquito, another mechanism that enables evasion of sequence-specific immune responses (Grubaugh, Weger-Lucarelli et al. 2016). Some ISVs interact with histone H4, a proviral factor, and

increase its expression, which upregulates the replication of other co-infecting viruses. (Olmo, Todjro et al. 2023).

### piRNA biogenesis in *Ae. aegypti*

piRNA biogenesis in *Ae. aegypti* is similar to that of *D. melanogaster* described in Chapter 1 of this thesis, however, the somatic piRNA pathway differs from the mainly germline pathway described in Drosophila. In *Ae. aegypti* the piRNA clusters in the soma tend to be uni-stranded, with a recent bioinformatic analysis identifying 90% of somatic piRNA clusters as uni-stranded, and enriched in nrEVEs, while germline-only clusters are dual-stranded (Crava, Varghese et al. 2021, Qu, Betting et al. 2023). Most core piRNA clusters, i.e., clusters expressed in both somatic and gonadal tissue, produce 1U primary piRNAs that preferentially associate with Piwi5; however, two clusters show a specific Piwi4 interaction (Qu, Betting et al. 2023). Transcription from somatic piRNA clusters in *Ae. aegypti* appears to occur mostly through transcriptional readthrough where RNA polymerase II transcription machinery is co-opted during active transcription of upstream genes (Qu, Betting et al. 2023). The piRNA precursors are exported to the cytoplasm, where they undergo processing into primary piRNAs via phased piRNA biogenesis, where PIWI proteins complexed with piRNA that acts as a guide, cleave long piRNA transcripts generating precursor piRNAs (Mohn, Handler et al. 2015, Gainetdinov, Colpan et al. 2018).

In the model insect *D. melanogaster*, no viral piRNAs (vpiRNAs) are expressed during experimental viral infection (Petit 2016), whereas in both *Ae. aegypti* and *Ae. albopictus*, viral infection has been shown to result in the production of vpiRNAs in whole adults (Hess, Prasad et al. 2011, Saldaña, Etebari et al. 2017, Wang, Jin et al. 2018) and in cell culture (Schnettler, Donald et al. 2013, Dietrich, Jansen et al. 2017, Varjak, Donald et al. 2017, Joosten, Miesen et al. 2019, Tassetto, Kunitomi et al. 2019).

Dicer2-deficient cells (a C6/36 cell line derived from *Ae. albopictus*) showed a vpiRNA response when infected with DENV, which resulted in slightly reduced viral load compared to Dicer2-competent cells (Scott, Brackney et al. 2010). Molecular investigations of PIWI knockout in Aag2 cell culture indicate that Piwi5 and Ago3 proteins form the ping-pong amplification cycle assembled by Veneno, a Tudor family protein, that results in the production of secondary piRNAs. (Miesen, Girardi et al. 2015, Joosten, Miesen et al. 2019). Interestingly, knockdown of Ago3 and Piwi5 in cell culture only slightly increased viral replication in infected cells, even though the associated vpiRNAs were depleted (Schnettler, Donald et al. 2013, Dietrich, Jansen et al. 2017, Tassetto, Kunitomi et al. 2019), while knockdown of Piwi4 has been associated with increased viral replication in Aag2 cell culture (Varjak, Donald et al. 2017, Varjak, Maringer et al. 2017).

## Endogenous viral elements

Endogenous viral elements (EVEs) are integrated viral fragments that form a memory bank of previous insect-virus encounters (Katzourakis and Gifford 2010). Among dipterans, both major aedine arboviral vectors, *Ae. aegypti* and *Ae. albopictus* genomes are significantly enriched for non-retroviral EVEs (nrEVEs), from which piRNAs are produced (Palatini, Miesen et al. 2017, Suzuki, Frangeul et al. 2017, Whitfield, Dolan et al. 2017).

The question of how EVEs form is still under investigation. EVEs have been found in both mammalian and insect genomes, and in both cases, all identified EVEs resulted from single transcripts, suggesting that it is viral transcripts that are integrated (Katzourakis and Gifford 2010). Most EVEs in *Ae. aegypti* consist of fragments derived from a wide variety of virus families, with rhabdoviruses and flaviviruses among the EVEs most highly represented in the mosquito genome. (Crava, Varghese et al. 2021).

During infection with RNA viruses, viral RNA is transformed into viral DNA (vDNA) by host retrotransposon-encoded reverse transcriptase, which is important for amplifying the antiviral siRNA pathway and has been found to provide long-lasting immunity (Goic, Vodovar et al. 2013, Goic, Stapleford et al. 2016, Poirier, Goic et al. 2018). Given that EVEs are preferentially associated with retrotransposons in actively expressed piRNA clusters, some theorize that these retrotransposons are involved in the "capture" of vDNA forms, though this is yet to be confirmed (Palatini, Miesen et al. 2017, Whitfield, Dolan et al. 2017),

with Piwi4 found to be associated with genome-encoded EVE-piRNAs (Tassetto, Kunitomi et al. 2019). EVE-piRNA clusters contain more than three non-retroviral EVEs (nrEVEs) on average, tend to be active in both somatic and germline tissues, and produce uni-strand 1U piRNAs (Crava, Varghese et al. 2021).

### EVE-derived piRNAs – a conserved adaptive immune system in *Ae. aegypti*?

Experimentally, the siRNA pathway has been shown to be indispensable for antiviral immunity in mosquitoes reviewed in (Samuel, Adelman et al. 2018). However, *Dicer2* mutant mosquitoes showed variable tolerance to infection, indicating a secondary immune mechanism at play (Samuel, Pohlenz et al. 2023). Recent studies have indicated a case for inherited immunity in both *D. melanogaster* and *Ae. aegypti*. (Mondotte, Gausson et al. 2020). This, coupled with a recent demonstration of the antiviral activity of cell fusing agent virus (CFAV)-derived piRNAs in *Ae*. *aegypti* ovaries (Suzuki, Baidaliuk et al. 2020), lends credence to the theory that the co-option of an expanded piRNA pathway in limiting the replication of cognate viruses *via* EVE-derived piRNAs presents an alternative heritable immune system in the yellow fever mosquito.

### CHAPTER AIMS

The goal of this project is to investigate the link between the piRNA pathway and heritable antiviral immunity in *Ae. aegypti*. This was done by insertion of a viral sequence into a highly expressed piRNA locus with the aim to generate viral piRNAs which would then limit the

replication of the virus during active infection. The antiviral nature of piRNAs was hotly disputed for over a decade, and has recently been confirmed in mosquito ovaries, where EVE-derived piRNAs were shown to limit the replication of the cognate virus (Suzuki, Baidaliuk et al. 2020). As viruses can be vertically transmitted, it is probable that a similarly designed antiviral system is employed by mosquitoes to limit the replication of both arboviruses and ISVs, which are both typically from the same virus families.

The goal of this project was to engineer a mosquito to express vpiRNAs targeted to a specific arbovirus. This immune activity would be heritable and systemic, rather than confined to the ovaries, and would limit the replication of the virus in the mosquito, especially at sites important for transmission, i.e., the midgut epithelium and the salivary glands. While vpiRNAs may not completely shut down viral replication, it may reduce the numbers so they're low enough to reduce the probability of transmission. First, however, successful CRISPR-Cas9 mutagenesis of a piRNA locus characterized by highly repetitive sequences was required. Here, the successful insertion of a plasmid containing a segment of SINV identified as a hot-spot of vpiRNAs, using CRISPR-Cas9 is shown. Manipulation of this locus represents the first step in engineering mosquito immunity to arboviruses.

## MATERIALS AND METHODS

### sgRNA design

To guide the choice of sgRNAs in a highly repetitive locus, unpublished data from a tissue-specific piRNA library were used. The sequences were mapped to the older *Ae. aegypti* Liverpool genome (AaegL3). The chosen piRNA locus was highly expressed in larval salivary glands and gastric caecae, tissues associated with viral infection and dissemination. To increase the probabilities of successful CRISPR-Cas9 editing, the chosen locus was remapped to the most current *Ae. aegypti* build (AaegL5) which was a more contiguous, chromosome-level assembly (Matthews, Dudchenko et al. 2018). This was done using NCBI Remap tool (https://www.ncbi.nlm.nih.gov/genome/tools/remap) with the Assembly-Assembly option using default parameters.

The chosen locus was found to exist in the current build and the sgRNAs were designed by hand as conventional sgRNA design programs did not handle repetitive regions well. The sgRNAs were checked for specificity using BLASTn in VectorBase (https://vectorbase.org/vectorbase/app/workspace/blast/) as well as off-target cleavage using Cas-OFFinder (http://www.rgenome.net/cas-offinder/). Successful sgRNAs (listed in Table 3.1) were purchased from Synthego (Redwood City, CA) with the standard chemical modifications.

## Virus choice

Sindbis virus (SINV) is a single-stranded RNA virus in the alphavirus genus that is transmitted Culicine mosquitoes and causes mild illness in humans. This virus was chosen as it is a BSL2 virus, as it causes mild disease in humans, and is widely used in mosquito infection studies both *in vivo* and *in vitro*. It has also been shown experimentally that alphaviruses consistently produce piRNAs in cell culture (Blair, Olson et al. 2019).

Experimental infections of *Ae. aegypti* cell culture (Aag2) with SINV showed that most vpiRNAs produced in response to infection are centered at a peak around 8000 bp, located at the start of the viral sub-genome in the capsid gene (Miesen, Girardi et al. 2015). Later infections in other mosquito cell lines representing *Ae. albopictus* (U4.4, C6/36) showed the same distribution of piRNAs. (Brackney, Scott et al. 2010, Vodovar, Bronkhorst et al. 2012, Miesen, Girardi et al. 2015). A 500 bp segment of this region from the virus was synthesized by Twist Bioscience (Q-101384). The identity of this fragment was confirmed by Sanger sequencing, following the submission guidelines (Retrogen Inc, San Diego, CA).

## Plasmid design and cloning

The CRISPaint-GFP plasmid (Addgene, Watertown, Massachusetts) was purchased and linearized by PCR using CPgfp primers followed by PCR purification using the QIAquick PCR Purification Kit (Qiagen , Venlo, The Netherlands). 20 bp of overhangs were added to the SINV fragment by PCR using sin primers, and the product was sent for Sanger

sequencing to confirm the correct sequence. Both the linearized plasmid and the SINV fragment were gel purified (Qiagen, Venlo , The Netherlands) in a 1% agarose gel before use to increase the chances of successful cloning. The plasmid assembly reaction was carried out using NEB HiFi, with the vector:insert ratio of 1:5 to give the final plasmid, named CRISPaint-SINV-GFP (CP-SIN), illustrated in Fig. 3.1.

The assembled plasmid was diluted 4x and transformed into JM109 competent cells (Zymo Research Corporation, Tustin, CA) which were then plated on LB plates with Ampicillin antibiotic selection marker. Single colonies were picked and mini-prepped (Zymo Research Corporation, Tustin, CA) and analyzed via restriction digestion using HincII (New England Biolabs, Ipswich, MA) following by resolving of the fragments on a 0.8% agarose gel. Samples showing the expected restriction digest pattern (two bands, 1474 bp and 3296 bp) were sequenced using M13F and M13R primers, to confirm the correct assembly of CP-SIN-GFP. All primer sequences can be found in Table 3.1.

### Injection mix and embryo microinjection and rearing

Embryo microinjection and rearing were carried out as described in Chapter 2 of this thesis. The 6 sgRNAs were first tested in 3 trails for efficacy *in vivo* by microinjection into the Exu strain with the CRISPaint-GFP plasmid and the frame-selector sgRNAs as follows to a total volume of 50 µl, then stored in aliquots of 3 µl at -80C.

| | |
|---|---|
| 6 piRNA locus sgRNAs | 100 ng/µl each |
| 3 CRISPaint Frame-selector sgRNAs | 100 ng/µl each |
| CRISPaint-GFP plasmid | 300 ng/µl |

Successful sgRNAs were used in the experimental injections with the modified plasmid CRISPaint-SIN-GFP. Cas9 (PNA Bio INC, Thousand Oaks, CA) was added to increase the rate of editing in a difficult region and the injection mix was made up as follows in 50 µl nuclease-free water:

| | |
|---|---|
| 4 piRNA locus sgRNAs | 100 ng/µl each |
| 3 CRISPaint frame-selector sgRNAs | 100 ng/µl each |
| CRISPaint-SIN-GFP plasmid | 300 ng/µl |
| Cas9 | 300 ng/µl |

Each sgRNA was complexed with an aliquot of Cas9 by incubation at 37°C for 5 minutes.

### Molecular analysis

G0 mosquitoes were reared to adulthood and pool-mated, unless GFP+ was found in the developing eye. Any mosquitoes that die during development are collected and frozen at -20°C for processing later. Once eggs are laid, the adults are sacrificed, and genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue kit (Qiagen, Venlo, The Netherlands) following manufacturer's recommendations for insect tissue, with the following exception, the insect tissue was lysed at 56°C overnight instead of 1-3 hours. The

gDNA was quantified using the Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) according to manufacturer's instructions.

For each individual G0 mosquito, the three piRNA cluster regions were amplified using Q5 High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and the PCR products were visualized on a 1% agarose gel. Successful amplicons were column purified (Qiagen, Venlo, The Netherlands) and quantified again using the Nanodrop 2000. Primers used to perform PCR can be found in Table 3.1.

To determine which mosquitoes were successfully edited, the cleaned PCR amplicons were subjected to T7 Endonuclease I (New England Biolabs, Ipswich, MA) which catalyzes the cleavage of mismatched DNA. This was done following manufacturer's protocol and the product was analyzed on a 1.5% agarose gel.

Samples with two or more bands in the T7EI assay were selected for cloning into the pJet1.2/blunt vector using the CloneJET PCR Cloning kit (Thermo Fisher Scientific Inc., Waltham, MA) following the manufacturer's instructions. The plasmid was then transformed into chemically competent JM109 cells (Zymo Research Corporation, Tustin, CA). The cells, which contain an ampicillin resistance selection marker are then plated onto LB agar with ampicillin and incubated for 16 hours overnight at 37°C. Single colonies were picked and outgrown in LB media with ampicillin for 16 hours overnight in a 37°C shaker. 1 µl of the liquid culture was used as a template for colony PCR with AccuPower Taq PCR PreMix (Bioneer, Inc., Oakland, CA) following the manufacturer's protocol, with an initial

cell lysis hold at 96°C for 6 minutes. The colony PCR positive samples, as confirmed on a 1% agarose gel, were prepared for Sanger sequencing at Retrogen Inc (San Diego, CA) following template submission guidelines with the pJet-For or pJet-Rev primers. See Fig. 3.2 for a schematic of the genomic context of the chosen locus in the piRNA cluster.

Sequencing files were viewed and manipulated using SnapGene (GSL Biotech LLC, San Diego, CA). The sequences were aligned against the *Ae. aegypti* reference genome using BLASTn in VectorBase as above, and indels at the sgRNA target sites were manually annotated.

### Next-generation amplicon sequencing of positive G0 mosquitoes

For G0 mosquitoes that were GFP+ at screening, amplicon sequencing was carried out (Azenta, Burlington, MA). Briefly, sequencing adapters were added to the target site amplicons via PCR using LocA1 EZ and LocB EZ primer sets (Table 3.1). The PCR product was column-purified as before and assessed for quality and quantity using the Qubit dsDNA BR kit and the Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA). The amplicons were submitted for NGS sequencing following the guidelines outlined by the manufacturer.

Analysis of the amplicon sequencing results was performed in Geneious Prime 2023.1.1 (Boston, MA). The paired reads for each positive G0 were imported into the program and trimmed using the BBDuk trimmer (version 38.84) in Geneious to remove the Illumina

adapters, low quality reads (minimum score set to 6), and short reads (less than 50 bp). The reads were set as paired reads then merged into one file using BBMerge (version 38.84). The "Analyze CRISPR editing results" operation was used on the merged reads with the options set to identify variants within the entire range covered by the reads.

## RESULTS

### Remapping a repetitive piRNA locus

The data used to inform the choice of target locus was originally mapped to the AaegL3 genome build, which was at the supercontig level. The assembly was especially patchy in repetitive regions of the genome such as piRNA loci. To accurately target a highly repetitive locus, the coordinates needed to be remapped to the new genome build (AaegL5). This is because the new build is highly contiguous and relatively well annotated in terms of transcriptomic data. The remapped data was used to identify piRNA loci with high expression in the larval midgut and salivary glands, as identified by small RNA sequencing in the Atkinson lab (Han 2017).

### Efficiency of sgRNAs targeting a repetitive region

Six sgRNAs targeting the chosen locus were designed and tested *in vivo*, with editing efficiency determined by next-generation amplicon sequencing. No indels were identified at sgRNA S4 or S6, and comparatively few indels were found at sgRNAs S3 and S5, therefore these sgRNA were not used in microinjections. This is crucial as a high injection load results

in lower injected embryo hatching frequencies, and so only S1 and S2 were chosen. Interestingly, these two sgRNAs are located in an unannotated gene (AAEL007844) with two putative domains identified in Uniprot (UniProt 2023), one of which is a transposase domain (Tra8) which is surrounded by a variety of genes and gene fragments (see Table 3.2). Table 3.3 shows the efficiency of all six sgRNAs in the three trial injections.

### Generation of G0 GFP-positive mosquitoes

Injected embryos were reared to adulthood as described before and observed for phenotypic differences during development. Further injections into the locus resulted in two GFP+ G0 mosquitoes, which both died soon after eclosing, therefore they were unable to be mated to potentially pass down the plasmid to the next generation (Table 3.4). Sanger sequencing revealed three additional mutant alleles (Fig. 3.3). Additional injections with only sgRNAs S1 and S2 were completed, though no further targeted gene editing was observed (Table 3.4).

The mutant alleles were either identified manually by blasting the sequence to the reference genome in VectorBase (Amos, Aurrecoechea et al. 2022) and looking for indels, or by utilizing a software known as ICE that infers edits from Sanger sequencing data (Conant, Hsiau et al. 2022) (Fig. 3.3).

## Confirmation of integration of the CRISPaint-GFP-SINV plasmid

G0 mosquitoes were marked positive if they displayed the characteristic GFP fluorescence in the developing eye, best seen in the pupal stage of development. Therefore, G0 and G1 pupae were screened for GFP fluorescence (Fig. 3.4). No GFP+ G1 mosquitoes were recovered, consistent with the lack of molecular evidence of inherited mutant alleles.

Amplification of the SINV segment in CP-SIN was performed on the gDNA of GFP+ mosquitoes, to confirm the presence of plasmid sequences, as this sequence is not found in the *Ae. aegypti* genome as confirmed by BLASTn analysis against the AaegL5 genome build (Matthews, Dudchenko et al. 2018). As the trial 3 GFP+ G0 mosquito was injected before the CP-SIN plasmid was assembled, it was not expected to show amplification, while the SINV segment is clearly present in the trial 4 GFP+ mosquito (Fig 3.5). The same PCR was carried out on the subsequent injection experiments as a screen for insertion of the plasmid without GFP expression, which occurred in injection experiment 7, shown in Fig. 3.6, with one G0 mosquito testing positive for the presence of the SINV sequence, and therefore part of the plasmid.

The graph in Fig. 3.7A illustrates the observations made in the trial injection , while the graph in Fig. 3.7B illustrates the same for the experimental injections. The insertion frequencies of the plasmid were between 0.12% to 0.25%, reflecting the difficulty of transforming a highly repetitive locus. The hatch frequencies of injected embryos were higher in the experimental

injections (Fig. 3.7B) though, there was greater variability in the percentage of embryos that hatched.

Amplicon sequencing for the experiment 3 injections GFP+ mosquito revealed 83 unique variants at the S5 sgRNA target site with the longest deletions recovered being a 19 bp and 23 bp deletion. At sgRNA S3, 26 unique variants were recovered from the same mosquito, with mostly 2 bp deletions. For the GFP+ mosquito from experiment 4 injections, 384 unique variants were called at sgRNA S3 with the reads mostly containing 1-2 bp deletions and insertions. The trial 3 GFP+ mosquito from the injections with the CRISPaint-GFP plasmid resulted in 491 unique variants with mostly 1-2 bp deletions and insertions.

## DISCUSSION

Mosquitoes are colloquially known as the world's most dangerous animal and are responsible for a large proportion of annual global morbidity and mortality (Prudêncio 2020). There is an urgent need to develop new vector control tools to supplement currently existing strategies. In the pursuit of this endeavor, a better understanding of the mosquito immune system is crucial, especially the interplay between Culicine mosquitoes and viruses that enable them to withstand a high viral load with little to no fitness cost (Lambrechts and Scott Thomas 2009).

During active infection with an RNA virus, it is reverse transcribed into viral DNA (vDNA) by endogenous transposable element activity, and the vDNA is then transcribed to form a

more diverse pool of viral siRNAs which target the infecting virus for degradation via RNAi (Poirier, Goic et al. 2018). This mechanism has been identified as allowing for persistent infection in cell culture, as it results in limited viral replication (Goic, Vodovar et al. 2013), and may be a step in the process of formation of new EVEs via integration of the vDNA (Poirier, Goic et al. 2018). Viral-derived sequences are occasionally integrated into the host genome leading to the formation of EVEs which then acquire a variety of functions, some immune-related (Takahashi, Heaton et al. 2021). Bioinformatic analysis of the Aag2 genome revealed an abundance of EVEs mostly from eight viral families, with majority of annotated EVEs derived from viruses in the Rhabdoviridae, Flaviviridae, and Chuviridae families (Whitfield, Dolan et al. 2017). Most EVEs are derived from insect specific viruses (ISVs) that were endogenized into the germline perhaps during transovarial transmission, a common method of transmission of ISVs, sometime in the mosquito's evolutionary history (Palatini, Miesen et al. 2017). Additionally, EVE-derived piRNAs have been shown to limit the replication of the cognate virus in *Ae. aegypti* ovaries (Suzuki, Baidaliuk et al. 2020). The successful use of CRISPR-Cas9 in mosquitoes facilitated the potential to knock-in an artificial EVE specific to an arbovirus of medical concern into an actively expressed piRNA cluster. Altogether, this represents an attractive opportunity to co-opt the mosquito's natural immune system for our gain by engineering immunity to SINV using a synthetic EVE.

More than half of the *Ae. aegypti* genome is composed of repetitive sequences and EVEs from which piRNAs are expressed (Matthews, Dudchenko et al. 2018). This makes the task of identifying and then targeting a piRNA cluster uniquely challenging (Fig. 3.8). However, careful design of sgRNAs makes this possible, though at low frequencies (Fig. 3.5). One of the most highly expressed piRNA loci in *Ae. aegypti*. (Arensburger, Hice et al. 2011, Han 2017, Aguiar, de Almeida et al. 2020) which is littered with EVEs was the piRNA cluster chosen. Experiments in both cell culture (Miesen, Girardi et al. 2015) and in the mosquito (Aguiar, de Almeida et al. 2020) confirm that this approximately 3 kb stretch of DNA, termed the flamenco-like locus for its similarity to the similarly named piRNA cluster *in D. melanogaster*, occurs on chromosome 2.

More importantly, the chosen piRNA cluster had high expression of piRNAs specifically in larval midgut and salivary gland tissues (Han 2017). These are tissues of interest to viral transmission as they provide the first opportunity for immune intervention. For a virus to be transmitted from one human host to another, the virus is ingested during a blood meal and infects the mosquito midgut epithelial lining, dissemination into the hemocoel, followed by infection of tissues including the salivary glands, where the virus accumulates ready to be transmitted to the next vertebrate host via mosquito saliva which is injected into host via a blood meal. (Danet, Beauclair et al. 2019)

The yellow fever mosquito's impressive vectorial capacity may be due to the complex and ever-evolving relationship between aedine mosquitoes and viruses where both sides race to limit the damage inflicted by the other. The piRNA pathway provides an elastic mechanism through which Ae. aegypti may be able to limit the replication of infecting arboviruses to tolerable levels. This can be tested by generating heritable immunity to a virus by induction of vpiRNA production from an artificial EVE. While i was not able to set up a line to test production of EVEs, in this work I demonstrated that it is possible to insert genetic cargo into a repetitive locus in the mosquito genome.

Molecular analysis of GFP+ G0 mosquitoes proved to be a challenge as well, due to the mosaic nature of the mosquitoes at the target sites. For example, while the SINV fragment was expected to be found in both G0 mosquitoes shown in Fig. 3.4B and 3.4C, PCR amplification of the fragment only showed a positive result with the G0 mosquito in 4C. Interestingly, the latter mosquito had GFP fluorescence in both eyes when observed at the pupal stage, while the pupa in 4B only had fluorescence in one eye, perhaps indicating that a smaller proportion of its cells were edited during embryo microinjections.

Next-generation amplicon sequencing was initially chosen to analyze the G0 mosquitoes as it should capture most of the alleles present in the mosaic without the need for sequencing single clones which can be tedious. However, a serious limitation of amplicon sequencing is the amplicon size limit of 450 bp, which is problematic when analyzing a multiplexed

CRISPR-Cas9 experiment where two sgRNAs are designed per target site. Designing an amplicon of 450 bp may lead to loss of information on larger deletions at the target site. Experiments in mouse and human cell culture have shown that sequencing a larger region around the target site reveals more complex editing events; including large deletions in 20% of edited cells of up to 6kb induced by one sgRNA sgRNA (Kosicki, Tomberg et al. 2018, Higashitani and Horie 2023, Park, Cao et al. 2023). Further injected mosquitoes were analyzed using the ICE program (Conant, Hsiau et al. 2022) which is more flexible in terms of the size of the amplicon. This is because the software accepts Sanger sequencing files and has an amplicon size recommendation of 400 – 800 bp, with one limitation to being that the primers need to be at least 150 bp away from the sgRNA target cut site, which required a redesign of the S3 and S4 primer pairs.

In *Ae. albopictus*, while antiviral piRNAs have yet to be confirmed, experiments consisting of infection assays with alphaviruses showed a piRNA response with involvement mainly from Piwi5, as inferred from expression data during active *Ae. albopictus* viral infection (Morazzani, Wiley et al. 2012, Marconcini, Hernandez et al. 2019). Infection assays with a flavivirus show earlier activation of the piRNA pathway with Piwi6 and Piwi4 involvement around 2 days post-infection (Marconcini, Hernandez et al. 2019).

Co-option of EVEs for other functions is prevalent in mammalian genomes, with most of these functions being antiviral in nature (Frank and Feschotte 2017). This provides an

interesting analogous case to the expanded piRNA pathway in *Ae. aegypti*. Deep sequencing of small RNAs in wild-caught mosquitoes demonstrates that EVEs are population-dependent (Crava, Varghese et al. 2021, Spadar, Phelan et al. 2021), further supporting the theory that these EVEs are acquired randomly during mosquito-virus interactions. This also cautions against the use of cell culture to characterize EVEs and EVE-derived piRNAs, as the data obtained would not necessarily be applicable in the field.

The complex interplay between ISVs and arboviruses means that this antiviral pathway merits further study, as some ISVs have been shown to modulate arboviral infection in the mosquito (Olmo, Todjro et al. 2023). By deepening our understanding of this antiviral pathway, we can combine the recent advancements in CRISPR technology to develop a lasting intervention of deadly mosquito-borne viruses that are expected to increase with the progression of climate change. This opens the door for potential targets in the quest to control arboviral diseases. However, the difficulty of targeting a piRNA cluster demonstrated in this work underscores the need for more optimized CRISPR technologies in mosquito vectors. I attempted to address this gap in the field in Chapter 4.

## CONCLUSION

I attempted to recapitulate a naturally occurring process of heritable immunity to viruses by employing the use of CRISPR-Cas9 genetic editing technologies. In the wild, mosquitoes consume voraciously during the larval stages in water, and as adults where they take both

blood and sugar meals. During this feeding they are exposed to a variety of pathogens including entomopathogenic viruses, some of which have a deleterious effect on the survival of the mosquito. During active infection, a chance integration of a viral DNA produced during viral replication into the germline genome may be facilitated by retrotransposon-encoded reverse transcriptases. This chance integration may generate piRNAs, producing slightly enhanced immunity to the infecting virus, which may be enough to ensure selection and fixation of this EVE in the local mosquito population.

While mutagenesis of a piRNA locus proved challenging, insertion of the CRISPaint-SINV-GFP plasmid was successful, albeit at low frequencies. Unfortunately, there was no inheritance of the GFP+ phenotype or genotype observed in these injection series, though further injections should yield successful germline editing and generation of a line of transgenic CRISPaint-SINV-GFP mosquitoes.

This would allow for the dissection of the mechanism of piRNA cluster initiation, if EVE-piRNAs are observed, as well as introducing the possibility of mosquitoes genetically engineered to have EVE-mediated immunity to arboviruses. This presents a potential tool in the arsenal against *Ae. aegypti* and the diseases it transmits.

BIBLIOGRAPHY

Adelman, Z. N., I. Sanchez-Vargas, E. A. Travanty, J. O. Carlson, B. J. Beaty, C. D. Blair and K. E. Olson (2002). "RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome." J. Virol. **76**(24): 12925-12933.

Aguiar, E. R. G. R., J. P. P. de Almeida, L. R. Queiroz, L. S. Oliveira, R. P. Olmo, I. J. d. S. de Faria, J.-L. Imler, A. Gruber, B. J. Matthews and J. T. Marques (2020). "A single unidirectional piRNA cluster similar to the flamenco locus is the major source of EVE-derived transcription and small RNAs in Aedes aegypti mosquitoes." RNA **26**(5): 581-594.

Amos, B., C. Aurrecoechea, M. Barba, A. Barreto, E. Y. Basenko, W. Bażant, R. Belnap, A. S. Blevins, U. Böhme, J. Brestelli, B. P. Brunk, M. Caddick, D. Callan, L. Campbell, M. B. Christensen, G. K. Christophides, K. Crouch, K. Davis, J. DeBarry, R. Doherty, Y. Duan, M. Dunn, D. Falke, S. Fisher, P. Flicek, B. Fox, B. Gajria, G. I. Giraldo-Calderón, O. S. Harb, E. Harper, C. Hertz-Fowler, M. J. Hickman, C. Howington, S. Hu, J. Humphrey, J. Iodice, A. Jones, J. Judkins, S. A. Kelly, J. C. Kissinger, D. K. Kwon, K. Lamoureux, D. Lawson, W. Li, K. Lies, D. Lodha, J. Long, R. M. MacCallum, G. Maslen, M. A. McDowell, J. Nabrzyski, D. S. Roos, S. S. C. Rund, S. W. Schulman, A. Shanmugasundram, V. Sitnik, D. Spruill, D. Starns, C. J. Stoeckert, S. S. Tomko, H. Wang, S. Warrenfeltz, R. Wieck, P. A. Wilkinson, L. Xu and J. Zheng (2022). "VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center." Nucleic Acids Res. **50**(D1): D898-D911.

Arensburger, P., R. H. Hice, J. A. Wright, N. L. Craig and P. W. Atkinson (2011). "The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs." BMC Genomics **12**: 606.

Besson, B., O. M. Lezcano, G. J. Overheul, K. Janssen, C. G. Spruijt, M. Vermeulen, J. Qu and R. P. van Rij (2022). "Arbovirus-vector protein interactomics identifies Loquacious as a co-factor for dengue virus replication in Aedes mosquitoes." bioRxiv.

Blair, C. D., K. E. Olson and M. Bonizzoni (2019). "The Widespread Occurrence and Potential Biological Roles of Endogenous Viral Elements in Insect Genomes." Curr. Issues Mol. Biol. **34**: 13-30.

Brackney, D. E., J. C. Scott, F. Sagawa, J. E. Woodward, N. A. Miller, F. D. Schilkey, J. Mudge, J. Wilusz, K. E. Olson, C. D. Blair and G. D. Ebel (2010). "C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response." PLoS Negl. Trop. Dis. **4**(10): e856.

Conant, D., T. Hsiau, N. Rossi, J. Oki, T. Maures, K. Waite, J. Yang, S. Joshi, R. Kelso, K. Holden, B. L. Enzmann and R. Stoner (2022). "Inference of CRISPR Edits from Sanger Trace Data." CRISPR J **5**(1): 123-130.

Crava, C. M., F. S. Varghese, E. Pischedda, R. Halbach, U. Palatini, M. Marconcini, L. Gasmi, S. Redmond, Y. Afrane, D. Ayala, C. Paupy, R. Carballar-Lejarazu, P. Miesen, R. P. van Rij and M. Bonizzoni (2021). "Population genomics in the arboviral vector Aedes aegypti reveals the genomic architecture and evolution of endogenous viral elements." Mol. Ecol. **30**(7): 1594-1611.

Danet, L., G. Beauclair, M. Berthet, G. Moratorio, S. Gracias, F. Tangy, V. Choumet and N. Jouvenet (2019). "Midgut barriers prevent the replication and dissemination of the yellow fever vaccine in Aedes aegypti." PLoS Negl. Trop. Dis. **13**(8): e0007299.

de Almeida, J. P., E. R. Aguiar, J. N. Armache, R. P. Olmo and J. T. Marques (2021). "The virome of vector mosquitoes." Curr. Opin. Virol. **49**: 7-12.

Dietrich, I., S. Jansen, G. Fall, S. Lorenzen, M. Rudolf, K. Huber, A. Heitmann, S. Schicht, E. H. Ndiaye, M. Watson, I. Castelli, B. Brennan, R. M. Elliott, M. Diallo, A. A. Sall, A.-B. Failloux, E. Schnettler, A. Kohl and S. C. Becker (2017). "RNA Interference Restricts Rift Valley Fever Virus in Multiple Insect Systems." mSphere **2**(3).

El Chamy, L., V. Leclerc, I. Caldelari and J.-M. Reichhart (2008). "Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll." Nat. Immunol. **9**(10): 1165-1170.

Frank, J. A. and C. Feschotte (2017). "Co-option of endogenous viral sequences for host cell function." Curr. Opin. Virol. **25**: 81-89.

Franz, A. W. E., A. M. Kantor, A. L. Passarelli and R. J. Clem (2015). "Tissue Barriers to Arbovirus Infection in Mosquitoes." Viruses **7**(7): 3741-3767.
Gainetdinov, I., C. Colpan, A. Arif, K. Cecchini and P. D. Zamore (2018). "A Single Mechanism of Biogenesis, Initiated and Directed by PIWI Proteins, Explains piRNA Production in Most Animals." Mol. Cell **71**(5): 775-790.e775.

Göertz, G. P., J. J. Fros, P. Miesen, C. B. F. Vogels, M. L. van der Bent, C. Geertsema, C. J. M. Koenraadt, R. P. van Rij, M. M. van Oers and G. P. Pijlman (2016). "Noncoding Subgenomic Flavivirus RNA Is Processed by the Mosquito RNA Interference Machinery and Determines West Nile Virus Transmission by Culex pipiens Mosquitoes." J. Virol. **90**(22): 10145-10159.

Goic, B., K. A. Stapleford, L. Frangeul, A. J. Doucet, V. Gausson, H. Blanc, N. Schemmel-Jofre, G. Cristofari, L. Lambrechts, M. Vignuzzi and M.-C. Saleh (2016). "Virus-derived DNA drives mosquito vector tolerance to arboviral infection." Nat. Commun. **7**: 12410.

Goic, B., N. Vodovar, J. A. Mondotte, C. Monot, L. Frangeul, H. Blanc, V. Gausson, J. Vera-Otarola, G. Cristofari and M.-C. Saleh (2013). "RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila." Nat. Immunol. **14**(4): 396-403.

Grubaugh, N. D., J. Weger-Lucarelli, R. A. Murrieta, J. R. Fauver, S. M. Garcia-Luna, A. N. Prasad, W. C. t. Black and G. D. Ebel (2016). "Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching." Cell Host Microbe **19**(4): 481-492.

Han, M. E. (2017). The piRNA System in Aedes aegypti. Ph.D. Ph.D. Thesis, University of California, Riverside.

Hess, A. M., A. N. Prasad, A. Ptitsyn, G. D. Ebel, K. E. Olson, C. Barbacioru, C. Monighetti and C. L. Campbell (2011). "Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense." BMC Microbiol. **11**: 45.

Higashitani, Y. and K. Horie (2023). "Long-read sequence analysis of MMEJ-mediated CRISPR genome editing reveals complex on-target vector insertions that may escape standard PCR-based quality control." Sci. Rep. **13**(1): 11652.

Joosten, J., P. Miesen, E. Taşköprü, B. Pennings, P. W. T. C. Jansen, M. A. Huynen, M. Vermeulen and R. P. Van Rij (2019). "The Tudor protein Veneno assembles the ping-pong amplification complex that produces viral piRNAs in Aedes mosquitoes." Nucleic Acids Res. **47**(5): 2546-2559.

Kakumani, P. K., S. S. Ponia, R. K. S, V. Sood, M. Chinnappan, A. C. Banerjea, G. R. Medigeshi, P. Malhotra, S. K. Mukherjee and R. K. Bhatnagar (2013). "Role of RNA interference (RNAi) in dengue virus replication and identification of NS4B as an RNAi suppressor." J. Virol. **87**(16): 8870-8883.
Katzourakis, A. and R. J. Gifford (2010). "Endogenous viral elements in animal genomes." PLoS Genet. **6**(11): e1001191.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat. Rev. Mol. Cell Biol. **10**(2): 126-139.

Kosicki, M., K. Tomberg and A. Bradley (2018). "Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements." Nat. Biotechnol. **36**(8): 765-771.

Lambrechts, L. and W. Scott Thomas (2009). "Mode of transmission and the evolution of arbovirus virulence in mosquito vectors." Proceedings of the Royal Society B: Biological Sciences **276**(1660): 1369-1378.

Lemaitre, B. and J. Hoffmann (2007). "The host defense of Drosophila melanogaster." Annu. Rev. Immunol. **25**: 697-743.

Li, W.-X., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. A. Brydon, K. L. Johnson, A. García-Sastre, L. A. Ball, P. Palese and S.-W. Ding (2004). "Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing." Proc. Natl. Acad. Sci. U. S. A. **101**(5): 1350-1355.

Liu-Helmersson, J., H. Stenlund, A. Wilder-Smith and J. Rocklöv (2014). "Vectorial capacity of Aedes aegypti: effects of temperature and implications for global dengue epidemic potential." PLoS One **9**(3): e89783.

Liu, Q. and Z. Paroo (2010). "Biochemical principles of small RNA pathways." Annu. Rev. Biochem. **79**: 295-319.

Marconcini, M., L. Hernandez, G. Iovino, V. Houé, F. Valerio, U. Palatini, E. Pischedda, J. E. Crawford, B. J. White, T. Lin, R. Carballar-Lejarazu, L. Ometto, F. Forneris, A.-B. Failloux and M. Bonizzoni (2019). "Polymorphism analyses and protein modelling inform on functional specialization of Piwi clade genes in the arboviral vector Aedes albopictus." PLoS Negl. Trop. Dis. **13**(12): e0007919.

Matthews, B. J., O. Dudchenko, S. B. Kingan, S. Koren, I. Antoshechkin, J. E. Crawford, W. J. Glassford, M. Herre, S. N. Redmond, N. H. Rose, G. D. Weedall, Y. Wu, S. S. Batra, C. A. Brito-Sierra, S. D. Buckingham, C. L. Campbell, S. Chan, E. Cox, B. R. Evans, T. Fansiri, I. Filipović, A. Fontaine, A. Gloria-Soria, R. Hall, V. S. Joardar, A. K. Jones, R. G. G. Kay, V. K. Kodali, J. Lee, G. J. Lycett, S. N. Mitchell, J. Muehling, M. R. Murphy, A. D. Omer, F. A. Partridge, P. Peluso, A. P. Aiden, V. Ramasamy, G. Rašić, S. Roy, K. Saavedra-Rodriguez, S. Sharan, A. Sharma, M. L. Smith, J. Turner, A. M. Weakley, Z. Zhao, O. S. Akbari, W. C. t. Black, H. Cao, A. C. Darby, C. A. Hill, J. S. Johnston, T. D. Murphy, A. S. Raikhel, D. B. Sattelle, I. V. Sharakhov, B. J. White, L. Zhao, E. L. Aiden, R. S. Mann, L. Lambrechts, J. R. Powell, M. V. Sharakhova, Z. Tu, H. M. Robertson, C. S. McBride, A. R. Hastie, J. Korlach, D. E. Neafsey, A. M. Phillippy and L. B. Vosshall (2018). "Improved reference genome of Aedes aegypti informs arbovirus vector control." Nature **563**(7732): 501-507.

Merkling, S. H. and R. P. van Rij (2013). "Beyond RNAi: antiviral defense strategies in Drosophila and mosquito." J. Insect Physiol. **59**(2): 159-170.

Miesen, P., E. Girardi and R. P. van Rij (2015). "Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in Aedes aegypti mosquito cells." Nucleic Acids Res. **43**(13): 6545-6556.

Mohn, F., D. Handler and J. Brennecke (2015). "Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis." Science **348**(6236): 812-817.
Mondotte, J. A., V. Gausson, L. Frangeul, Y. Suzuki, M. Vazeille, V. Mongelli, H. Blanc, A.-B. Failloux and M.-C. Saleh (2020). "Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects." Cell Rep. **33**(11): 108506.

Morazzani, E. M., M. R. Wiley, M. G. Murreddu, Z. N. Adelman and K. M. Myles (2012). "Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma." PLoS Pathog. **8**(1): e1002470.

Myles, K. M., D. J. Pierro and K. E. Olson (2003). "Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti." J. Virol. **77**(16): 8872-8881.

Olmo, R. P., Y. M. H. Todjro, E. R. G. R. Aguiar, J. P. P. de Almeida, F. V. Ferreira, J. N. Armache, I. J. S. de Faria, A. G. A. Ferreira, S. C. G. Amadou, A. T. S. Silva, K. P. R. de Souza, A. P. P. Vilela, A. Babarit, C. H. Tan, M. Diallo, A. Gaye, C. Paupy, J. Obame-Nkoghe, T. M. Visser, C. J. M. Koenraadt, M. A. Wongsokarijo, A. L. C. Cruz, M. T. Prieto, M. C. P. Parra, M. L. Nogueira, V. Avelino-Silva, R. N. Mota, M. A. Z. Borges, B. P. Drumond, E. G. Kroon, M. Recker, L. Sedda, E. Marois, J.-L. Imler and J. T. Marques (2023). "Mosquito vector competence for dengue is modulated by insect-specific viruses." Nat Microbiol **8**(1): 135-149.

Palatini, U., P. Miesen, R. Carballar-Lejarazu, L. Ometto, E. Rizzo, Z. Tu, R. P. van Rij and M. Bonizzoni (2017). "Comparative genomics shows that viral integrations are abundant and express piRNAs in the arboviral vectors Aedes aegypti and Aedes albopictus." BMC Genomics **18**(1): 512.
Paradkar, P. N., J.-B. Duchemin, R. Voysey and P. J. Walker (2014). "Dicer-2-dependent activation of Culex Vago occurs via the TRAF-Rel2 signaling pathway." PLoS Negl. Trop. Dis. **8**(4): e2823.
Paradkar, P. N., L. Trinidad, R. Voysey, J.-B. Duchemin and P. J. Walker (2012). "Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway." Proc. Natl. Acad. Sci. U. S. A. **109**(46): 18915-18920.

Park, S. H., M. Cao and G. Bao (2023). "Detection and quantification of unintended large on-target gene modifications due to CRISPR/Cas9 editing." Current Opinion in Biomedical Engineering **28**: 100478.

Poirier, E. Z., B. Goic, L. Tomé-Poderti, L. Frangeul, J. Boussier, V. Gausson, H. Blanc, T. Vallet, H. Loyd, L. I. Levi, S. Lanciano, C. Baron, S. H. Merkling, L. Lambrechts, M. Mirouze, S. Carpenter, M. Vignuzzi and M.-C. Saleh (2018). "Dicer-2-Dependent Generation of Viral DNA

from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects." Cell Host Microbe **23**(3): 353-365.e358.

Qu, J., V. Betting, R. van Iterson, F. M. Kwaschik and R. P. van Rij (2023). "Chromatin profiling identifies transcriptional readthrough as a conserved mechanism for piRNA biogenesis in mosquitoes." Cell Rep. **42**(3): 112257.

Salazar, M. I., J. H. Richardson, I. Sánchez-Vargas, K. E. Olson and B. J. Beaty (2007). "Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes." BMC Microbiol. **7**: 9.

Saldaña, M. A., K. Etebari, C. E. Hart, S. G. Widen, T. G. Wood, S. Thangamani, S. Asgari and G. L. Hughes (2017). "Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes." PLoS Negl. Trop. Dis. **11**(7): e0005760.

Samuel, G. H., Z. N. Adelman and K. M. Myles (2018). "Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes." Trends Microbiol. **26**(5): 447-461.

Samuel, G. H., T. Pohlenz, Y. Dong, N. Coskun, Z. N. Adelman, G. Dimopoulos and K. M. Myles (2023). "RNA interference is essential to modulating the pathogenesis of mosquito-borne viruses in the yellow fever mosquito Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **120**(11): e2213701120.

Sánchez-Vargas, I., J. C. Scott, B. K. Poole-Smith, A. W. E. Franz, V. Barbosa-Solomieu, J. Wilusz, K. E. Olson and C. D. Blair (2009). "Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway." PLoS Pathog. **5**(2): e1000299.

Schnettler, E., C. L. Donald, S. Human, M. Watson, R. W. C. Siu, M. McFarlane, J. K. Fazakerley, A. Kohl and R. Fragkoudis (2013). "Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells." J. Gen. Virol. **94**(Pt 7): 1680-1689.
Scott, J. C., D. E. Brackney, C. L. Campbell, V. Bondu-Hawkins, B. Hjelle, G. D. Ebel, K. E. Olson and C. D. Blair (2010). "Comparison of dengue virus type 2-specific small RNAs from RNA interference-competent and -incompetent mosquito cells." PLoS Negl. Trop. Dis. **4**(10): e848.

Sim, S., J. L. Ramirez and G. Dimopoulos (2012). "Dengue virus infection of the Aedes aegypti salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior." PLoS Pathog. **8**(3): e1002631.

Souza-Neto, J. A., S. Sim and G. Dimopoulos (2009). "An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense." Proc. Natl. Acad. Sci. U. S. A. **106**(42): 17841-17846.

Spadar, A., J. E. Phelan, E. D. Benavente, M. Campos, L. F. Gomez, F. Mohareb, T. G. Clark and S. Campino (2021). "Flavivirus integrations in Aedes aegypti are limited and highly conserved across

samples from different geographic regions unlike integrations in Aedes albopictus." Parasit. Vectors **14**(1): 332.

Suzuki, Y., A. Baidaliuk, P. Miesen, L. Frangeul, A. B. Crist, S. H. Merkling, A. Fontaine, S. Lequime, I. Moltini-Conclois, H. Blanc, R. P. van Rij, L. Lambrechts and M.-C. Saleh (2020). "Non-retroviral endogenous viral element limits cognate virus replication in Aedes aegypti ovaries." Curr. Biol.

Suzuki, Y., L. Frangeul, L. B. Dickson, H. Blanc, Y. Verdier, J. Vinh, L. Lambrechts and M.-C. Saleh (2017). "Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes." J. Virol. **91**(15).

Tassetto, M., M. Kunitomi, Z. J. Whitfield, P. T. Dolan, I. Sánchez-Vargas, M. Garcia-Knight, I. Ribiero, T. Chen, K. E. Olson and R. Andino (2019). "Control of RNA viruses in mosquito cells through the acquisition of vDNA and endogenous viral elements." Elife **8**.

UniProt, C. (2023). "UniProt: the Universal Protein Knowledgebase in 2023." Nucleic Acids Res. **51**(D1): D523-D531.

Varjak, M., C. L. Donald, T. J. Mottram, V. B. Sreenu, A. Merits, K. Maringer, E. Schnettler and A. Kohl (2017). "Characterization of the Zika virus induced small RNA response in Aedes aegypti cells." PLoS Negl. Trop. Dis. **11**(10): e0006010.

Varjak, M., K. Maringer, M. Watson, V. B. Sreenu, A. C. Fredericks, E. Pondeville, C. L. Donald, J. Sterk, J. Kean, M. Vazeille, A.-B. Failloux, A. Kohl and E. Schnettler (2017). "Aedes aegypti Piwi4 Is a Noncanonical PIWI Protein Involved in Antiviral Responses." mSphere **2**(3).

Vodovar, N., A. W. Bronkhorst, K. W. R. van Cleef, P. Miesen, H. Blanc, R. P. van Rij and M.-C. Saleh (2012). "Arbovirus-derived piRNAs exhibit a ping-pong signature in mosquito cells." PLoS One **7**(1): e30861.

Wang, Y., B. Jin, P. Liu, J. Li, X. Chen and J. Gu (2018). "piRNA Profiling of Dengue Virus Type 2-Infected Asian Tiger Mosquito and Midgut Tissues." Viruses **10**(4).

Weaver, S. C. and W. K. Reisen (2010). "Present and future arboviral threats." Antiviral Res. **85**(2): 328-345.

Whitfield, Z. J., P. T. Dolan, M. Kunitomi, M. Tassetto, M. G. Seetin, S. Oh, C. Heiner, E. Paxinos and R. Andino (2017). "The Diversity, Structure, and Function of Heritable Adaptive Immunity Sequences in the Aedes aegypti Genome." Curr. Biol. **27**(22): 3511-3519.e3517.

Xi, Z., J. L. Ramirez and G. Dimopoulos (2008). "The Aedes aegypti toll pathway controls dengue virus infection." PLoS Pathog. **4**(7): e1000098.

Xiao, X., Y. Liu, X. Zhang, J. Wang, Z. Li, X. Pang, P. Wang and G. Cheng (2014). "Complement-related proteins control the flavivirus infection of Aedes aegypti by inducing antimicrobial peptides." PLoS Pathog. **10**(4): e1004027.

# TABLES

| sgRNAs | Name | Sequence | Notes |
|---|---|---|---|
| S1 and S2 sgRNAs | SP For1 | GCATAGCACAGCCGTCATG | PCR primers |
| | SP Rev1 | CAGCTGTGACTGACACAAATAAC | |
| | SiPi gRNA 1 | AACAAGGCCAAGGCCGCTCG**CGG** | sgRNAs with PAM in red |
| | SiPi gRNA 2 | CGGCAAGTTTCCGGAGGGCA**CGG** | |
| | | | |
| S3 and S4 sgRNAs | LocB gRNA 1 | ATTTCGACTTTGGCAAGATA**GGG** | sgRNAs with PAM in red |
| | LocB gRNA 2 | TTAAGGTCGTGGATGTTGAC**AGG** | |
| | LocB FOR2 | CATCCATATCGGCCGACGAA | PCR primers |
| | LocB REV | AGGCAAGGAGTCAACCTTCG | |
| | LocB FOR | TGTTCCCGACAACAGAGTCG | amplicon sequencing primers |
| | LocB REV | AGGCAAGGAGTCAACCTTCG | |
| | LocB-EZ-For | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATCCATATCGGCCGACGAA | amplicon sequencing primers with adapters |
| | LocB-EZ-Rev | GACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGCAAGGAGTCAACCTTCG | |
| | | | |
| S5 and S6 sgRNAs | LocA gRNA 1 | TGACGAGGTCCACGATCCCG**AGG** | sgRNAs with PAM in red |
| | LocA gRNA 2 | GCCGTTCGTGCACGAGACCA**AGG** | |
| | LocA FOR2 | CAAATCGGCATTGAGGCTGG | PCR primers |
| | LocA REV2 | CCACCTGTTGCCCTCCATAG | |
| | LocA FOR | TGCAGGGTTCTCGGTTTGAA | amplicon sequencing primers |
| | LocA REV | CTCTGTGCTCACTGGTCAGG | |
| | LocA1-EZ-For | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCAGGGTTCTCGGTTTGAA | amplicon sequencing primers with adapters |
| | LocA1-EZ-Rev | GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCTGTGCTCACTGGTCAGG | |
| | | | |
| plasmid | SINV1 For | GCAACTAGACCTCAACCCCC | CRISPaint-SINV-GFP diagnostic primers |
| | SINV2 Rev | TACTCCGCGAGGGATGGTAA | |
| | M13F | TGTAAAACGACGGCCAGT | Plasmid primers |
| | M13R | TGTAAAACGACGGCCAGT | |

**Table 3.1:** Sequences of all sgRNAs and primers used in chapter 3 experiments.

| | GENE ID (Accession no.) | Function/Identity | Source | Additional comments |
|---|---|---|---|---|
| A | AAEL007854 – pelota | Translation release factor | N/A | Adham, I. M. et al. (2003) |
| B | AAEL028221 | No information | | |
| C | AAEL025868 | No information | | Spatacsin protein family |
| D | AAEL007853 | lupus la protein (Sjogren syndrome type B antigen) | | Putative nucleic acid binding/RNA processing |
| E | AAEL007850 | No information | | Nucleic acid binding |
| F | AAEL021610 (MT913594.1) | Flaviviral nonstructural protein | +ve strand RNA virus | Orthologue in *Aedes albopictus* |
| G | AAEL026638 | No information | | |
| H | AAEL003355 | No information | | Zinc finger protein family |
| I | AAEL003352 | 60S ribosomal protein l7ae | | |
| J | AAEL003347 | CRAL/TRIO domain family | | Retinaldehyde-binding |

**Table 3.2:** List of genes and gene fragments found in the target piRNA locus surrounding the sgRNA target AAEL007844 as well as functions that could be identified. See Fig. 3.6 for more information.

| Trial | No. eggs injected | Larvae | Hatch frequency (%) | Pupae | Adults | Ecclosion frequency (%) | Survival frequency from larvae (%) | GFP+ | Sequence confirmed | Transformation rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 779 | 56 | 7.2 | 44 | 43 | 97.7 | 76.8 | - | - | - |
| 2 | 652 | 24 | 3.7 | 22 | 20 | 90.9 | 83.3 | - | - | - |
| 3 | 826 | 73 | 8.8 | 72 | 71 | 98.6 | 97.3 | 1F | 1F | 0.12 |

**Table 3.3:** Injections to test sgRNA editing efficiency *in vivo*. The injection mix contained CRISPaint-GFP plasmid at 300ng/µl, FS 0,1,2 sgRNAs at 100ng/µl each S1+S2+S3+S5 sgRNAs at 100 ng/µl each (total 400ng/µl).

| Experiment | No. eggs injected | Larvae | Hatch frequency (%) | Pupae | Adults | Ecclosion frequency (%) | Survival frequency from larvae (%) | GFP+ | Sequence confirmed | Transformation rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 210 | 9 | 4.3 | 9 | 8 | 88.9 | 88.9 | - | - | - |
| 2 | 90 | 23 | 25.6 | 17 | 16 | 94.1 | 69.6 | - | - | - |
| 3 | 395 | 17 | 4.3 | 7 | 7 | 100 | 41.2 | 1M | 1M | 0.25 |
| 4 | 635 | 57 | 8.98 | 47 | 44 | 93.6 | 77.2 | 1F | 1F | 0.16 |
| 5 | 482 | 33 | 6.85 | 33 | 24 | 72.73 | 72.73 | - | 1 | - |
| 6 | 399 | 56 | 14.04 | 43 | 37 | 86.05 | 66.07 | - | 2 | - |
| 7 | 188 | 29 | 15.43 | 28 | 23 | 82.14 | 79.31 | - | - | - |

**Table 3.4:** The injection mix for experiments 1-5 contained CRISPaint-SINV-GFP plasmid at 300ng/µl, FS0,1,2 sgRNAs at 100ng/µl each S1+S2+S3+S4+S5+S6 sgRNAs at 100ng/µl each (total 600ng/µl), while in experiments 6-7, the only piRNA sgRNAs retained in the injection mix are S1 and S2.

## FIGURES

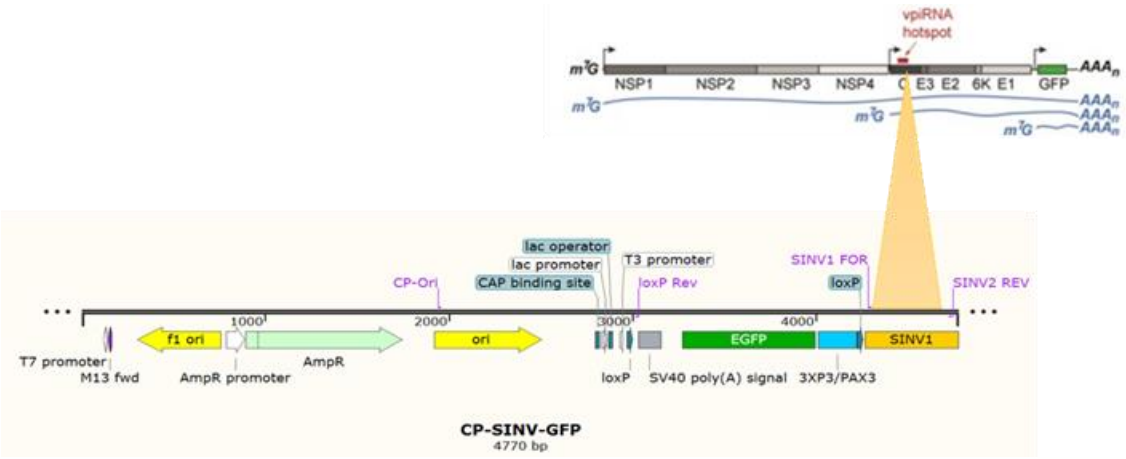


**Figure 3.1**: Schematic of the CRISPaint-SINV-GFP plasmid showing the viral piRNA (vpiRNA) hot spot identified by Miesen et al 2015.

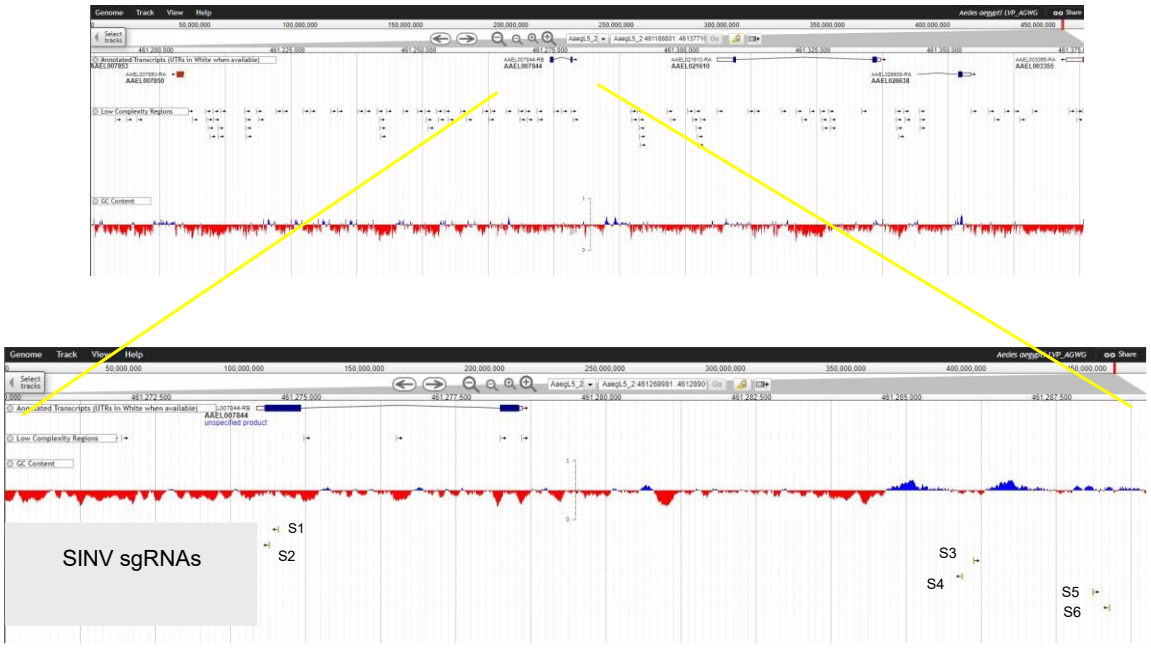


**Figure 3.2 :** Schematic showing the location of the six sgRNAs in the piRNA locus (the labelled arrows). The red histogram shows genomic regions with a GC content of less than 50% while the blue histogram shows regions with a GC content greater than 50%. The piRNA locus is littered with repetitive elements as shown by the black arrows. The sgRNAs represented as black arrows, were selected to be used in further injections based on the efficiency of the test injections.


**Figure 3.3:** Mutant alleles recovered from G0 mosquitoes in injection experiments 5 and 6 with CRISPR-Cas9 directed editing at S1 (3A) and S2 (3B) sgRNAs.

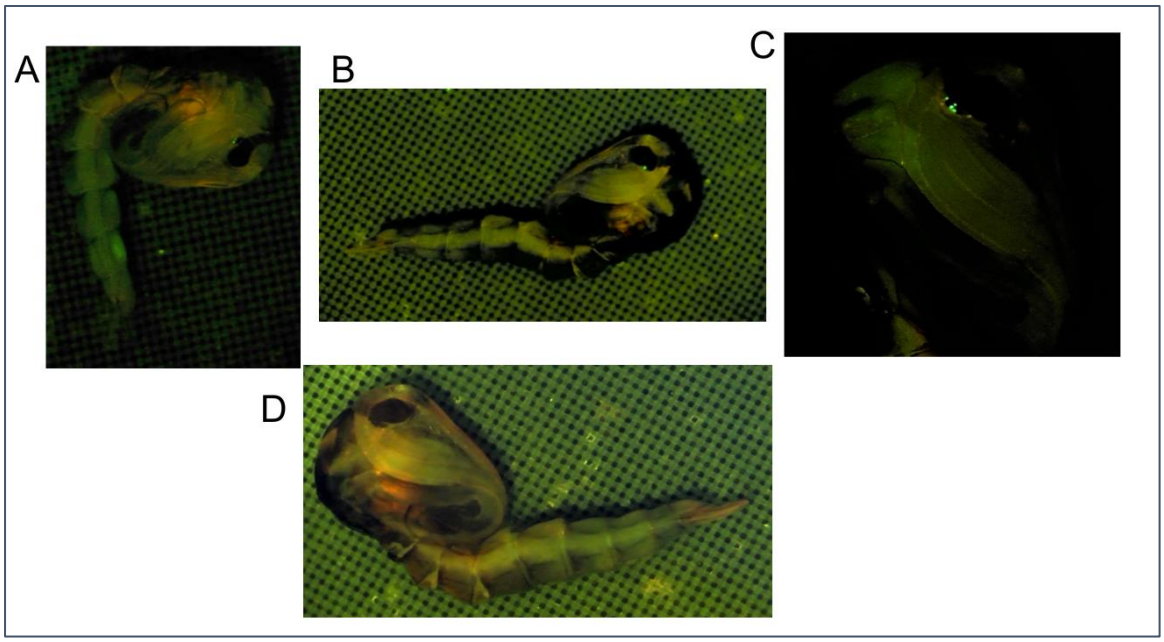


**Figure 3.4:** GFP+ G0 mosquitoes under fluorescent photography. **4A**: From injection trial 3, where the injection mix consisted of the CRISPaint-GFP plasmid at 300ng/µl, FS0,1,2 sgRNAs at 100ng/µl each,S1-S6 sgRNAs at 100ng/µl each. **4B** and **4C**: From injection experiment 3 and 4 respectively, where the injection mix consisted of CRISPaint-SINV-GFP plasmid at 300ng/µl, FS0,1,2 sgRNAs at 100ng/µl each S1+S2+S3+S4+S5+S6 sgRNAs at 100ng/µl each (total 600ng/µl). **4D**: Image of the eye without GFP fluorescence in the mosquito produced in experiment 3 injections (4B) which only had fluorescence in one eye when screened at the pupal stage.

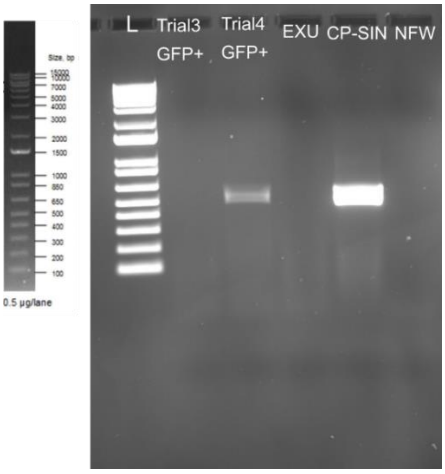


**Figure 3.5:** Gel image of PCR amplification of the SINV segment from the GFP+ G0 mosquitoes. The trial 3 injections were done with the CRISPaint-GFP plasmid; therefore, it was not expected to show an amplification. The trial 4 injections were done with the CRISPaint-GFP-SIN (CP-SIN) plasmid, hence the presence of the SINV segment. The Exu-Cas9 background strain into which injections are carried out did not contain the SINV segment as previously confirmed. The CP-SIN plasmid served as a positive control, with nuclease-free water (NFW) serving as the negative control.

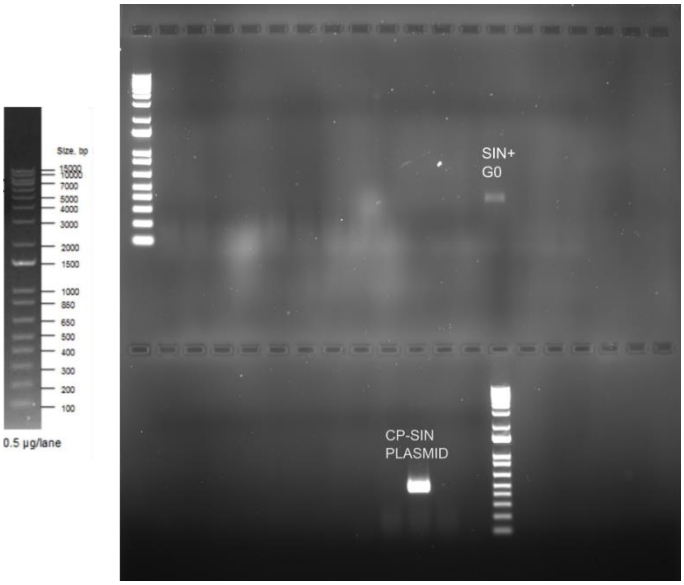


**Figure 3.6**: Gel image of PCR amplification of the SINV segment from the experiment 7 G0 mosquitoes. None of the pupae were positive for GFP expression in the eye. However, one mosquito (SIN+ G0) was positive for presence of the SINV fragment due to insertion of the CP-SIN plasmid.

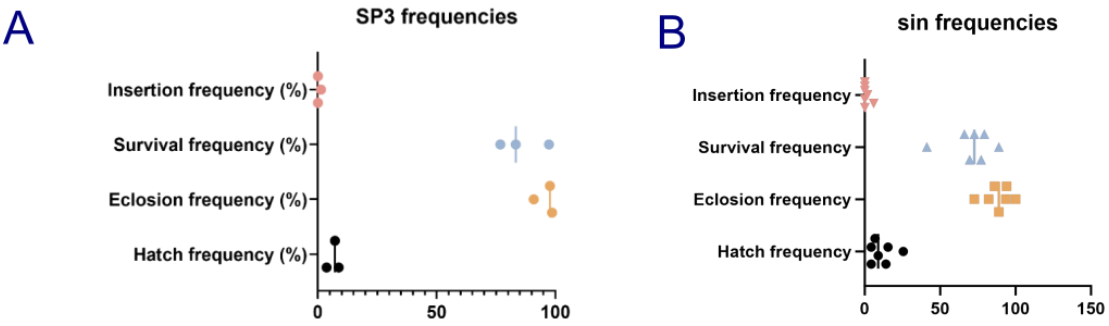


**Figure 3.7:** Graphical representation of the results of **7A**: the trial injections with the CRISPaint-GFP plasmid (SP3) and, **7B**: the experimental injections with the CRISPaint-GFP-SIN plasmid (sin).

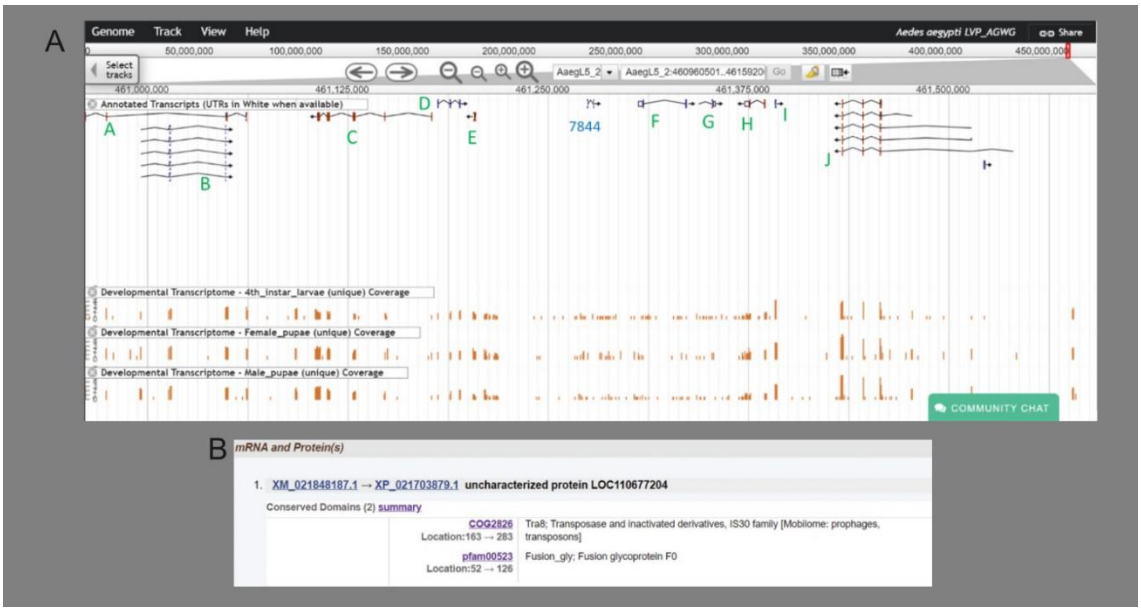


**Figure 3.8:** Genomic context of the target locus. **8A** Schematic of the target locus including the location and characteristics of the region surrounding AAEL007844. The other transcripts are expanded on in table 3. **8B** snapshot of Uniprot.org entry for AAEL007844 indicating two conserved domains: Tra8 (Transposase and inactive derivatives) and Fusion_gly (fusion glycoprotein).

# CHAPTER FOUR

## THE CRISPAINT TECHNICAL EXPERIMENT

## INTRODUCTION AND BACKGROUND

### A brief history of relevant genetic engineering in mosquitoes

While the main aim of heritable genetic engineering in mosquitoes is vector control, genetic perturbations are also a useful tool in the exploration of mosquito biology which can only enhance attempts at disease eradication (Terenius, Marinotti et al. 2008).

Transposable elements (TEs) were the popular tool of choice for insect transgenesis around the turn of the millennium (reviewed in (O'Brochta and Atkinson 1996, Handler 2001) with the yellow fever mosquito first successfully transformed in the late 1990s using a modified *Hermes* TE originally found in houseflies (*Musca domestica*) (Jasinskiene, Coates et al. 1998). A modified *mariner* element *Mos1* isolated from *Drosophila mauritiana* was also shown to be effective in *Ae. aegypti* (Coates, Jasinskiene et al. 1998) while *minos,* another TE derived from a Drosophilidae *D. hydei* was used to stably transform *An. stephensi* (Catteruccia, Nolan et al. 2000). *piggyBac,* a TE initially isolated from lepidopteran *Trichoplusia ni* was first shown to be effective in both *Ae. aegypti* and *An. gambiae* in 2001 after which it became the popular choice for transgenesis due to its transposition mechanism involving a precise insertion recognition sequence TTAA , additionally it excises precisely, leaving no scar, while

Hermes showed a tendency towards non-canonical integrations (Jasinskiene, Coates et al. 1998, Grossman, Rafferty et al. 2001, Kokoza, Ahmed et al. 2001, Lobo, Hua-Van et al. 2002).

While TEs proved to be successful in terms of generating stable lines, the somewhat random insertion into the genome and limited genetic load carrying capacity makes insertions difficult to fully characterize, as well as introducing variability due to position effects determined by the integration site (Handler 2001). TEs are still a part of the genetic engineering toolbox in use today, mainly employed in the creation of stable lines expressing CRISPR components for further experimentation (Zhu, Albishi et al. 2021).

Early gene editing tools such as zinc-finger nucleases (ZFNs) saw limited but successful use in *Ae. aegypti* to study mosquito physiology by mutating different sensory receptors (Degennaro et al 2013, McMeniman et al 2014, Corfas et al 2015).Transcription activator-like effector nucleases (TALENs) were another early gene editing tool that was successfully utilized in both *Ae. aegypti* and *An. gambiae* (Aryan, Anderson et al. 2013, Smidler, Terenzi et al. 2013). Continued use of ZFNs and TALENs petered off due to a few disadvantages including the relatively higher cost of reagents compared to the novel CRISPR-Cas9 system that was unveiled around the same time, as well as the labor and cost required to tailor the nucleases to new targets and organisms (Huang, Liu et al. 2016).

## CRISPR-Cas9 editing in *Ae. aegypti*

The co-option of the RNA-guided bacterial genetic defense system for use in genetic engineering of a wide variety of organisms (Doudna and Charpentier 2014) accelerated the pace of gene editing experiments in the yellow fever mosquito (Basu, Aryan et al. 2015, Dong, Lin et al. 2015), greatly aided by the publishing of a detailed description of a protocol to generate mutants using microinjection of CRISPR-Cas9 components into pre-blastoderm embryos (Kistler, Vosshall et al. 2015). The detailed protocol published by Kistler et al, revolutionized the field of genetic engineering in mosquitoes, providing a step-by-step guide which allowed other groups to successfully edit different targets with little optimization (reviewed in (Overcash and Adelman 2016).

Modifications to the initial published protocol have been published, with the highest editing rates seen when a genetically encoded Cas9 mosquito strain is crossed with a strain expressing sgRNAs (Zhu, Albishi et al. 2021, Sun, Li et al. 2022). Additional modifications include a technique known as Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control), in which Cas9 is fused to a peptide (P2C) which then facilitates the transport of the attached cargo, Cas9 RNP in this case, from the hemolymph into the ovaries, allowing for thoracic injection of blood-fed females instead of embryos (Chaverra-Rodriguez, Macias et al. 2018). Direct parental (DIPA) CRISPR employs a similar concept, with thoracic injections of the Cas9-RNP complex 24 hours after blood-feeding, taking advantage of the uptake of proteins

and other yolk components from the hemolymph during oocyte development (Shirai, Takahashi et al. 2023).

For the endonuclease to be targeted to the correct locus in the genome while minimizing off-target effects, the sgRNA must be unique, especially in the "seed" region, and it must be immediately upstream of a PAM site (Jinek, Chylinski et al. 2012). Cas9 activity results in a double-stranded break (DSB) in the DNA, 3 bp upstream of the PAM site (Kistler, Vosshall et al. 2015). Repair of the DSB proceeds through two main pathways, non-homologous end-joining (NHEJ) and homology-directed repair (HDR), where NHEJ is comparatively error prone and may result in the introduction of random indels during repair, while HDR uses the homologous chromosome as a template for faithful repair (Chiruvella, Liang et al. 2013, Ceccaldi, Rondinelli et al. 2016). An alternative form of NHEJ, known as microhomology-mediated end-joining (MMEJ) or alt-NHEJ uses local homology within around 30 base pairs as a template for repair, resulting in the deletion of the original target site (McVey and Lee 2008, Scully, Panday et al. 2019).

These, together with commonly used promoters to drive fluorescence as positive markers for transgenesis in mosquitoes (Bottino-Rojas and James 2022) have enabled rapid and relatively effective germline gene editing in *Ae. aegypti* as well as seven other mosquito species of medical importance (reviewed in (Riabinina, Quinn et al. 2022).

## CRISPaint

CRISPR-assisted insertion tagging (CRISPaint) is a technique first developed in human cell culture that uses NHEJ to insert DNA fragments into a specific site (Schmid-Burgk, Höning et al. 2016). NHEJ, while more error-prone, is the more attractive DSB repair strategy due to its occurrence more rapidly and at a higher frequency than HDR (Scully, Panday et al. 2019). Attempts to silence the NHEJ repair pathway resulted in higher frequencies of HDR, though this requires the addition of dsRNA targeting key NHEJ genes to the microinjection mix (Basu, Aryan et al. 2015). The CRISPaint system attempts to side-step the inefficient HDR pathway which requires unique homology arms, replaced by sgRNA donors, which direct Cas9 to the genomic target site as well as the plasmid target site, allowing for the use of a universal plasmid for different target loci (Bosch, Colbeth et al. 2019).

CRISPaint is a useful technique for tagging genes using fluorescent proteins to study native proteins *in vivo*, by inserting the fluorescent gene at the N or C terminal of the target protein, enabling more accurate characterizations of endogenous protein expression, when compared to the classical method of characterizing protein expression by overexpression (Shi, Kopparapu et al. 2023). CRISPaint also allows for the insertion of cargo in frame utilizing frame-selecting sgRNAs (FS sgRNAs), giving flexibility over the translation consequences for the target gene (Bosch, Colbeth et al. 2019). This technique has been used *in vivo* in *D.*

*melanogaster* and *in vitro*, namely with mouse embryonic stem cells and drosophila cells. (Bosch, Colbeth et al. 2019, Bosch, Knight et al. 2020, Shi, Kopparapu et al. 2023).

## CHAPTER AIMS

High frequency site-specific tools that enable insertion of genetic cargo into the germline are highly desirable tools in the global attempt to eradicate mosquito-borne diseases. CRISPaint represents a modular, easily programmable technology to insert large genetic loads while exploiting the more rapid and higher frequency NHEJ DSB repair pathway. This technology allows one to use a universal plasmid for each target site, cutting down on the time-consuming task of designing and making the homology arms which would be required for each target genomic location with traditional HDR.

Due to the reliance on the error prone NHEJ repair pathway, and the insertion of the entire plasmid into the target locus, CRISPaint is not a suitable technology to use with the goal of releasing the transgenic insects. However, more efficient genetic engineering tools would allow for the use of reverse genetics to expand the field's knowledge of vector biology, specifically reproductive behaviors as most population suppression approaches currently under trial are predicated on the release of males that can successfully compete with wild-type males in the field for mates (Shaw and Catteruccia 2019).

This technique has not yet been attempted in mosquitoes, to the best of my knowledge, therefore, the investigation of the efficiency of CRISPaint compared to traditional HDR would be useful for the mosquito genetic engineering community.

## MATERIALS AND METHODS

### Mosquito strain, sgRNAs and plasmid generation

CRISPaint-GFP (130277) and CRISPaint-RFP (130276) universal donor plasmids were purchased from Addgene as bacterial stabs (Watertown, MA). These plasmids, generated for use in *D. melanogaster*, encode a fluorescent protein, either GFP or dsRed under the control of the 3XP3/Pax3 promoter (Bosch, Colbeth et al. 2019). Both the frame-selector sgRNAs, and the genomic target loci sgRNAs were purchased from Synthego (Redwood, California). Table 4.1 shows the sgRNAs and PCR primers used in this experiment, as well as the expected size of the PCR amplicon.

The mosquito strain used for microinjections was Exu-Cas9, a transgenic strain derived from the Liverpool strain that expresses Cas9 under the control of germline promoter *Exuperentia* (Li, Bui et al. 2017), and was a kind gift of Dr. Omar Akbari (UCSD).

The plasmids were prepared by maxiprep using the Zyppy plasmid maxiprep kit following the manufacturer's directions (Zymo Research, Irvine, CA). The plasmids were then

sequenced via Sanger sequencing to confirm the sequence before use in the microinjection mix (Retrogen Inc, San Diego, CA).

Injection mixes were made with three different CRISPaint plasmid concentrations (100 ng/µl, 300 ng/µl, and 500 ng/µl) for each target gene as follows:

| | |
|---|---|
| 2 genomic locus sgRNAs | 100 ng/µl each |
| 3 CRISPaint Frame-selector sgRNAs | 100 ng/µl each |
| CRISPaint-GFP plasmid | 100/300/500 ng/µl |

### Microinjection, screening, and rearing

Microinjection was carried out following (Kistler, Vosshall et al. 2015) with modifications from (Li, Bui et al. 2017) with approximately 150 embryos injected in triplicate for each plasmid concentration tested. Microinjection and rearing were carried out according to the protocol elaborated on in chapter 2 of this thesis The G0 mosquitoes were screened visually both for the presence of the mosaic knock-out phenotype as well as the fluorescent marker, then pooled in a maximum of two mosquitoes/tubes for molecular screening for the presence of indels at the target site via PCR using the Q5 high-fidelity polymerase (New England Biolabs, Ipswich, MA) and T7 endonuclease I (T7E1) assay (New England Biolabs, Ipswich, MA) described in greater detail in chapter 2.

## RESULTS

### WHITE

The experiment targeting the *white* gene was repeated due to poor hatching seen in the first set of injections. There was no evidence of insertion of the plasmid inferred by the lack of GFP fluorescence seen in the eye of any G0 or G1 mosquito, therefore molecular analysis of the G0 mosquitoes was not done. Table 4.2 shows the first *white* experiment, where the highest hatch frequency was 7.06% in the W5A injection group. A total of 16 G0 mosquitoes displaying the mosaic *white* deletion phenotype, with examples shown in Fig. 4.1 were observed, giving an editing rate of 1.09% phenotypically positive mosquitoes, when compared to the total number of embryos injected.

Table 4.3 shows the second set of *white* injections with improved hatching where the highest hatch frequency was 16.1%. A total of 19 G0 pupae displayed the mosaic *white* deletion phenotype, giving a slightly higher editing rate of 1.28%, perhaps reflecting better insectary conditions.

### YELLOW

The hatch frequencies in the Yellow CRISPaint experiment, shown in Table 4.4 were lower than expected, with the highest seen in Y5C at 16.05%. Few G0 mosquitoes were mosaic for the *yellow* deletion phenotype, with a total of 5 out of 1419 injected embryos. However, the

*yellow* mutant phenotype was unexpectedly difficult to screen especially in the G0 mosquitoes, as mosaicism in the cuticle color is difficult to discern, meaning that the actual editing rate may be higher than 0.35% seen in this experiment. Fig. 4.2 shows examples of pupae that appeared to have a more yellow colored cuticle, though the difference is difficult to photograph. Due to these challenges and the lack of evidence of insertion of the plasmid, molecular analysis of the G0 mosquitoes was not done.

EBONY

Table 4.5 shows the injection data for the Ebony CRISPaint experiment. While the hatching frequencies of the injected embryos were low, as seen in the other experiments, there was evidence of insertion of the plasmid shown in fig. 4.3E. The positive pupa is shown under brightfield in the panel on the left, and GFP fluorescence can be seen in the eye under the GFP filter in the panel on the right. The GFP positive pupa came from the 500ng/µl plasmid concentration experiment, specifically E5B, reflecting the higher probability of integration with higher plasmid concentration in the injection mix.

The PCR and T7E1 assay results in Fig. 4.4 illustrate that the sgRNAs were effective. Cleavage by both sgRNAs would result in a mixture of fragments of approximately 250 bp and 460 bp with the Ae-Ebony-1 sgRNA and 550 bp and 160 bp with the Ae-Ebony-2 sgRNA, as evidenced in the PCR and T7E1 assay images in Fig. 4.4. The colony PCR results

(Fig. 4.4) after cloning of the PCR amplicon shown in the red rectangle on the PCR gel, are an example of the multiple alleles present in the pooled sample of two mosquitoes.

When determining the editing frequencies in the Ebony CRISPaint experiment, the presence of multiple bands in the PCR amplicon was interpreted as evidence of editing along with a positive T7E1 assay result. The results, summarized in Table 4.6, show that there were no PCR or T7E1 assay positive mosquitoes in the 300ng/μl plasmid concentration experiment, however 25% were PCR positive and 37.5% of pools were T7E1 positive at 500ng/μl plasmid concentration. At 100 ng/μl CRISPaint plasmid concentration, the editing frequencies were lower, with 13.3% of pools positive in both PCR and T7E1 assays.

## DISCUSSION

The CRISPaint technical experiments were designed to determine the efficiency of CRISPaint as a technology for genome editing in *Ae. aegypti* compared to the published data on the relatively more traditional homology-directed repair. The frequency of successful integration of genetic cargo in mosquitoes using the HDR repair pathway following CRISPR-Cas9 mediated cleavage is low compared to the knockout frequencies due to the NHEJ repair pathway. CRISPaint takes advantage of this by knocking in a plasmid using the NHEJ pathway resulting in higher integration rates compared to traditional HDR (Bosch, Colbeth et al. 2019).

The target genes, *white*, *ebony*, and *yellow*, were chosen based on published visible knock-out phenotypes to facilitate ease of screening and identifying potential transgenics (Li, Bui et al. 2017). Three different plasmid concentrations (100 ng/µl, 300 ng/µl and 500 ng/µl) were tested in triplicate to determine recommendations for optimized transformation experiments in the yellow fever mosquito. Different concentrations were tested as increased injection load increasing the probability of death in the injected embryos, a concern that needs to be balanced against the potential for increased integration events with higher plasmid concentrations.

Approximately, 150 embryos per replicate were injected and reared to adulthood. The G0 mosquitoes were screened for the appropriate mosaic knock-out phenotype and the presence of GFP fluorescence in the fourth larval stage as well as in the pupal stage. Each replicate group was self-mated and the G1 progeny screened for the knock-out phenotype as well as presence of the CRISPaint-GFP plasmid.

The hatch frequencies and insertion frequencies as evidenced by the presence of fluorescence in the developing eye of the pupae were comparatively low, with an integration rate of 0.59% in ebony injection group E5B, compared to published HDR insertion frequencies in *Ae. aegypti* which range from approximately 0.15% - 3.5% (Kistler, Vosshall et al. 2015, Li, Bui et al. 2017, Ang, Nevard et al. 2022). This was likely due to the fluctuations in humidity conditions in the insectary where the mosquitoes were held. The optimal humidity at which

mosquitoes are reared reflects the humidity in the regions with abundant mosquitoes, with lab conditions held between 60% - 80% relative humidity.

While the conditions under which the mosquitoes were reared were not ideal, successful insertion of the plasmid was achieved with the Ebony-CRISPaint-GFP experiment (Fig. 4.3), showing that CRISPaint is indeed effective in *Ae. aegypti*. Another factor that may limit the frequency of successful integration is the source used to provide the female mosquitoes a bloodmeal, with blood provided by live anesthetized mice shown to give higher frequencies of injected eggs (Sun, Li et al. 2022).

This presents a relatively rapid technique to insert large genetic cargo into the *Ae. aegypti* genome with higher frequencies than traditional HDR methods, albeit with one major limitation, which is that the entire plasmid may be incorporated into the genome (Bosch, Colbeth et al. 2019). While this makes CRISPaint unsuitable for genetic engineering when the goal is the eventual release of the transgenic insect, it is a useful tool to study yellow fever mosquito biology with the aim for better vector control. One example of a case in which a better system to insert genetic cargo is necessary is discussed in Chapter 3.

## CONCLUSION

The hatch frequencies of injected embryos for these experiments were significantly lower than expected. This was probably due to the lack of constant ideal humidity levels experienced by the mosquitoes, which reduces the overall fitness of the mosquitoes.

Regardless of the technical challenges faced in this experiment, CRISPaint is effective in *Ae. aegypti*, though the system may require further optimization for higher insertion frequencies.

As the graph in Table 4.6 shows, increasing the plasmid concentration increases the frequency of successful plasmid integrations using the CRISPaint system. There are likely upper limits to plasmid concentration in the injection mix due to the possibility for the mix to become viscous and clog the microinjection needle, the probability of toxic effects resulting in lower hatching and survival of injected mosquitoes. There is also the prohibition of the cost of procuring the plasmid in large amounts that would need to be considered. It would take repeating this experiment and testing higher plasmid concentration to further optimize CRISPaint in *Ae. aegypti*.

BIBLIOGRAPHY

Aryan, A., M. A. E. Anderson, K. M. Myles and Z. N. Adelman (2013). "TALEN-based gene disruption in the dengue vector Aedes aegypti." PLoS One **8**(3): e60082.

Basu, S., A. Aryan, J. M. Overcash, G. H. Samuel, M. A. E. Anderson, T. J. Dahlem, K. M. Myles and Z. N. Adelman (2015). "Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **112**(13): 4038-4043.

Bosch, J. A., R. Colbeth, J. Zirin and N. Perrimon (2019). "Gene knock-ins in Drosophila using homology-independent insertion of universal donor plasmids." bioRxiv.

Bosch, J. A., S. Knight, O. Kanca, J. Zirin, D. Yang-Zhou, Y. Hu, J. Rodiger, G. Amador, H. J. Bellen, N. Perrimon and S. E. Mohr (2020). "Use of the CRISPR-Cas9 System in Drosophila Cultured Cells to Introduce Fluorescent Tags into Endogenous Genes." Curr. Protoc. Mol. Biol. **130**(1): e112.

Bottino-Rojas, V. and A. A. James (2022). "Use of Insect Promoters in Genetic Engineering to Control Mosquito-Borne Diseases." Biomolecules **13**(1).

Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, C. Savakis, F. C. Kafatos and A. Crisanti (2000). "Stable germline transformation of the malaria mosquito Anopheles stephensi." Nature **405**(6789): 959-962.

Ceccaldi, R., B. Rondinelli and A. D. D'Andrea (2016). "Repair Pathway Choices and Consequences at the Double-Strand Break." Trends Cell Biol. **26**(1): 52-64.

Chaverra-Rodriguez, D., V. M. Macias, G. L. Hughes, S. Pujhari, Y. Suzuki, D. R. Peterson, D. Kim, S. McKeand and J. L. Rasgon (2018). "Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing." Nat. Commun. **9**(1): 3008.

Chiruvella, K. K., Z. Liang and T. E. Wilson (2013). "Repair of double-strand breaks by end joining." Cold Spring Harb. Perspect. Biol. **5**(5): a012757.

Coates, C. J., N. Jasinskiene, L. Miyashiro and A. A. James (1998). "Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3748-3751.

Dong, S., J. Lin, N. L. Held, R. J. Clem, A. L. Passarelli and A. W. E. Franz (2015). "Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti." PLoS One **10**(3): e0122353.

Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science **346**(6213): 1258096.

Grossman, G. L., C. S. Rafferty, J. R. Clayton, T. K. Stevens, O. Mukabayire and M. Q. Benedict (2001). "Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element." Insect Mol. Biol. **10**(6): 597-604.

Handler, A. M. (2001). "A current perspective on insect gene transformation." Insect Biochem. Mol. Biol. **31**(2): 111-128.

Huang, Y., Z. Liu and Y. S. Rong (2016). "Genome Editing: From Drosophila to Non-Model Insects and Beyond." J. Genet. Genomics **43**(5): 263-272.

Jasinskiene, N., C. J. Coates, M. Q. Benedict, A. J. Cornel, C. S. Rafferty, A. A. James and F. H. Collins (1998). "Stable transformation of the yellow fever mosquito, Aedes aegypti, with the Hermes element from the housefly." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3743-3747.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science **337**(6096): 816-821.

Kistler, K. E., L. B. Vosshall and B. J. Matthews (2015). "Genome engineering with CRISPR-Cas9 in the mosquito Aedes aegypti." Cell Rep. **11**(1): 51-60.

Kokoza, V., A. Ahmed, E. A. Wimmer and A. S. Raikhel (2001). "Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[3xP3-EGFP afm]." Insect Biochem. Mol. Biol. **31**(12): 1137-1143.

Li, M., M. Bui, T. Yang, C. S. Bowman, B. J. White and O. S. Akbari (2017). "Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **114**(49): E10540-E10549.

Lobo, N. F., A. Hua-Van, X. Li, B. M. Nolen and M. J. Fraser, Jr. (2002). "Germ line transformation of the yellow fever mosquito, Aedes aegypti, mediated by transpositional insertion of a piggyBac vector." Insect Mol. Biol. **11**(2): 133-139.

McVey, M. and S. E. Lee (2008). "MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings." Trends Genet. **24**(11): 529-538.

O'Brochta, D. A. and P. W. Atkinson (1996). "Transposable elements and gene transformation in non-drosophilid insects." Insect Biochem. Mol. Biol. **26**(8-9): 739-753.

Overcash, J. and Z. N. Adelman (2016). Chapter One - Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. Advances in Insect Physiology. A. S. Raikhel, Academic Press. **51:** 1-35.

Riabinina, O., M. Quinn and J. P. Whitehead (2022). "Genetic Toolbox Approaches in Mosquitoes." Cold Spring Harb. Protoc. **2022**(11): Pdb.top107691.

Schmid-Burgk, J. L., K. Höning, T. S. Ebert and V. Hornung (2016). "CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism." Nat. Commun. **7**: 12338.

Scully, R., A. Panday, R. Elango and N. A. Willis (2019). "DNA double-strand break repair-pathway choice in somatic mammalian cells." Nat. Rev. Mol. Cell Biol. **20**(11): 698-714.

Shaw, W. R. and F. Catteruccia (2019). "Vector biology meets disease control: using basic research to fight vector-borne diseases." Nat Microbiol **4**(1): 20-34.

Shi, Y., N. Kopparapu, L. Ohler and D. J. Dickinson (2023). "Efficient and rapid fluorescent protein knock-in with universal donors in mouse embryonic stem cells." Development **150**(10).

Shirai, Y., M. Takahashi, M. Ote, H. Kanuka and T. Daimon (2023). "DIPA-CRISPR gene editing in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae)." Appl. Entomol. Zool. **58**(3): 273-278.

Smidler, A. L., O. Terenzi, J. Soichot, E. A. Levashina and E. Marois (2013). "Targeted mutagenesis in the malaria mosquito using TALE nucleases." PLoS One **8**(8): e74511.

Sun, R., M. Li, C. J. McMeniman and O. S. Akbari (2022). "CRISPR-Mediated Genome Engineering in Aedes aegypti." Methods Mol. Biol. **2509**: 23-51.

Terenius, O., O. Marinotti, D. Sieglaff and A. A. James (2008). "Molecular genetic manipulation of vector mosquitoes." Cell Host Microbe **4**(5): 417-423.

Zhu, G.-H., N. M. Albishi, X. Chen, R. L. Brown and S. R. Palli (2021). "Expanding the Toolkit for Genome Editing in a Disease Vector, Aedes aegypti: Transgenic Lines Expressing Cas9 and Single Guide RNA Induce Efficient Mutagenesis." CRISPR J **4**(6): 846-853.

## TABLES

| | | |
|---|---|---|
| sgRNAs | Ae Yellow-1 sgRNA | GGCCAGTCACCGTGAATTCA |
| | Ae Yellow-2 sgRNA | GAGCAGGGTGGTGCCTGGATAGG |
| PCR primers | Ae Yellow For2 | GATGACCCACACGTTCTCTT |
| | Ae Yellow Rev2 | AATAAGCAGGAAATTGGCGTG |
| PCR amplicon size | | 931 bp |
| | | |
| sgRNAs | Ae Ebony-1 sgRNA | GGCCGTGTTCGGCGCAACGC |
| | Ae Ebony-2 sgRNA | CCTCGTTGCAGATGACAGCCG |
| | Ae Ebony-3 sgRNA | GACGGCCAACCGCGTGGCGGCGG |
| | Ae Ebony-4 sgRNA | ATGTAATCTCCGTCCCGATTCGG |
| PCR primers | Ae Ebony For2 | GCCGCTGACTATACGATTGAT |
| | Ae Ebony Rev4 | GCTCTACACCAGTAGAGACG |
| PCR amplicon size | | 717 bp |
| | | |
| sgRNAs | White sgRNA1 | GTCCTTCAGGTCGCCTCCGG |
| | White sgRNA2 | GGTCAGGGTTTCGGATGCAA |
| PCR primers | White For | TAAGAGGCAAGACACCACAAG |
| | White Rev | GCACGAACGACTCACATACA |
| PCR amplicon size | | 900 bp |

**Table 4.1:** Primers and sgRNAs used in the CRISPaint experiment targeting white, ebony, and yellow genes. The expected amplicon size is also included.

| | 100ng/µl | | | 300ng/µl | | | 500ng/µl | | |
|---|---|---|---|---|---|---|---|---|---|
| | **W1A** | **W1B** | **W1C** | **W3A** | **W3B** | **W3C** | **W5A** | **W5B** | **W5C** |
| **Injected eggs** | 157 | 162 | 153 | 161 | 160 | 161 | 170 | 170 | 173 |
| **Hatched** | 8 | 1 | 1 | 4 | 2 | 2 | 12 | 7 | 5 |
| **% hatched** | 5.10% | 0.62% | 0.65% | 2.49% | 1.25% | 1.24% | 7.06% | 4.12% | 2.89% |
| **White** | 2 | - | 1 | 2 | 2 | 2 | 2 | 4 | 1 |
| **GFP** | - | - | - | - | - | - | - | - | - |
| **G1 phenotype** | - | - | - | - | - | - | - | - | - |

**Table 4.2:** Injection table showing the results of the White CRISPaint-GFP experiment. Three injection mixes were used, each contained White sgRNA1, White sgRNA2, frame-selector sgRNAs 0, 1 and 2, and CRISPaint-GFP plasmid at one of three different concentrations –100ng/µl , 300ng/µl and 500ng/µl. For each concentration, approximately 150 embryos were injected with three replicates. The G1 progeny were also screened for fluorescence and the *white* mutant phenotype.

| | 100ng/µl | | | 300ng/µl | | | 500ng/µl | | |
|---|---|---|---|---|---|---|---|---|---|
| | **W1A** | **W1B** | **W1C** | **W3A** | **W3B** | **W3C** | **W5A** | **W5B** | **W5C** |
| Injected eggs | 187 | 138 | 180 | 161 | 167 | 162 | 156 | 152 | 185 |
| Hatched | 23 | 8 | 29 | 1 | 17 | 9 | 13 | 4 | 4 |
| % hatched | 12.3 % | 5.8% | 16.1% | * | 10.2% | 5.6% | 8.3% | 2.6% | 2.2% |
| White | 4 | 2 | 2 | - | 4 | 3 | 2 | 1 | 1 |
| GFP | - | - | - | - | - | - | - | - | - |

**Table 4.3:** Repeat of the white-CRISPaint-GFP experiment. The hatch frequencies are slightly higher than the first attempt, but lower than typical, and there was no evidence of insertion of the plasmid in any of the G0 or G1 mosquitoes.

| | 100ng/µl | | | 300ng/µl | | | 500ng/µl | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Y1A** | **Y1B** | **Y1C** | **Y3A** | **Y3B** | **Y3C** | **Y5A** | **Y5B** | **Y5C** |
| **Injected eggs** | 165 | 156 | 151 | 155 | 156 | 154 | 153 | 167 | 162 |
| **Hatched** | 1 | 9 | 14 | 4 | 1 | 2 | 16 | 8 | 26 |
| **% hatched** | 1.54% | 5.77% | 9.27% | 2.58% | 0.64% | 1.30% | 10.46% | 4.79% | 16.05% |
| **Yellow** | 1 | - | 1 | - | 1 | - | 2 | - | - |
| **GFP** | - | - | - | - | - | - | - | - | - |
| **G1 phenotype** | - | - | - | - | - | - | - | - | - |

**Table 4.4:** Injection table showing the results of the Yellow CRISPaint experiment. Three injection mixes were used, each contained Ae Yellow-1 sgRNA, Ae Yellow-2 sgRNA, frame-selector sgRNAs 0, 1 and 2, and CRISPaint-GFP plasmid at one of three different concentrations –100ng/µl, 300ng/µl and 500ng/µl. For each concentration, approximately 150 embryos were injected with three replicates. The G1 progeny were also screened for fluorescence and the *yellow* mutant phenotype.

| | 100ng/µl | | | 300ng/µl | | | 500ng/µl | | |
|---|---|---|---|---|---|---|---|---|---|
| | **E1A** | **E1B** | **E1C** | **E3A** | **E3B** | **E3C** | **E5A** | **E5B** | **E5C** |
| **Injected eggs** | 162 | 157 | 158 | 161 | 162 | 155 | 152 | 168 | 174 |
| **Hatched** | 4 | 14 | 5 | 2 | 4 | 3 | 9 | 12 | 20 |
| **% hatched** | 2.5% | 8.9% | 3.2% | 1.2% | 2.5% | 1.9% | 5.9% | 7.1% | 11.5% |
| **Ebony** | 1 | 2 | - | - | - | - | 3 | 1 | 4 |
| **GFP** | - | - | - | - | - | - | - | 1 | - |

**Table 4.5:** Injection table showing the results of the Ebony CRISPaint experiment. Three injection mixes were used, each contained Ae-Ebony sgRNA1&2, frame-selector sgRNAs 0, and CRISPaint-GFP plasmid at one of three different concentrations –100ng/µl, 300ng/µl and 500ng/µl. For each concentration, approximately 150 embryos were injected with three replicates. The G1 progeny were also screened for fluorescence and the *ebony* mutant phenotype. GFP fluorescence in the eye was observed in one pupa from injection group E5B.

| Ebony experiment | PCR positive | T7E1 assay positive | Sex |
|---|---|---|---|
| Ebony at 500 ng/µl (E-5) | 6/24 pools (25%) | 9/24 pools (37.5%) | All males |
| Ebony at 100 ng/µl (E-1) | 2/15 pools (13.3%) | 2/15 pools (13.3%) | 2 females; 2 males |

**Table 4.6:** Summary of Ebony-CRISPaint G0 positive mosquitoes. While there were no editing events captured in the Ebony at 300ng/µl (E-3) experiment, in the E-5 experiment 25% of all tested pools were PCR positive, while 37.5% of tested pools were positive in the T7E1 assay. In the E-1 experiment, 13.3% of all tested pooled G0 mosquitoes were positive in both the PCR and T7E1 assays.

## FIGURES

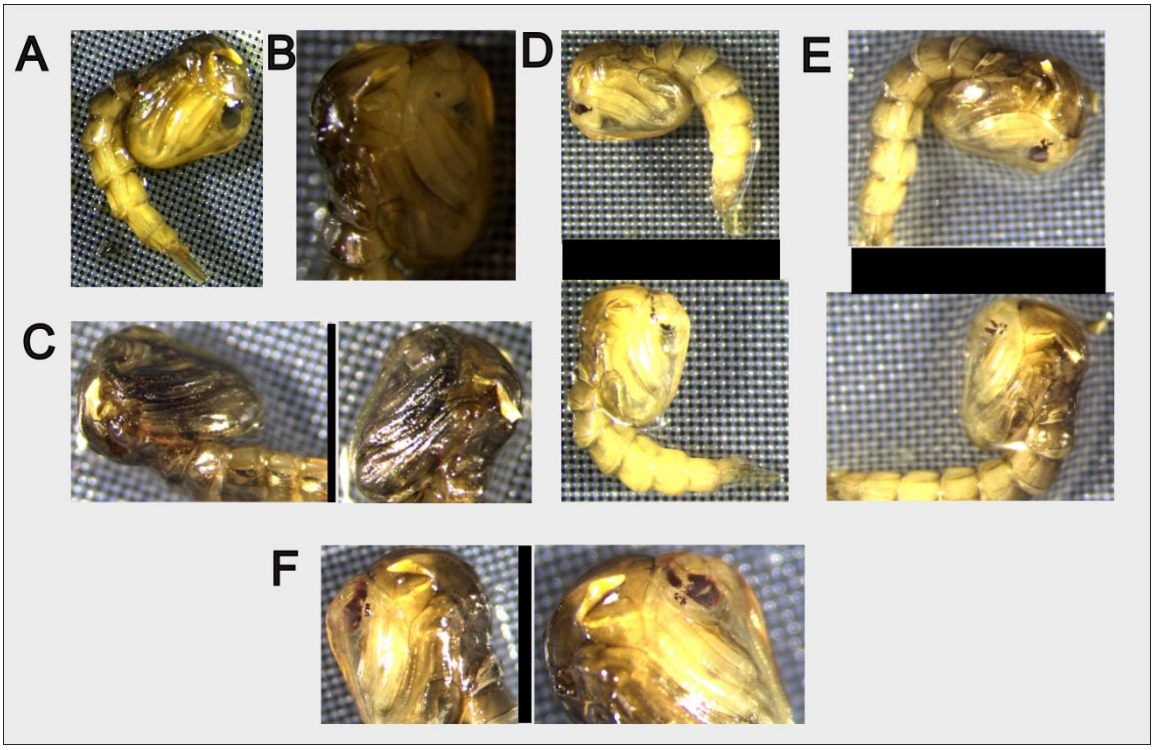


**Figure 4.1:** Examples of G0 pupae mosaic for mutation of the *white* gene. **A** – wild-type (WT) pupa, **B** – W3B, **C** – W5C, **D** – W5B, **E** – W3C, **F** – W5B.

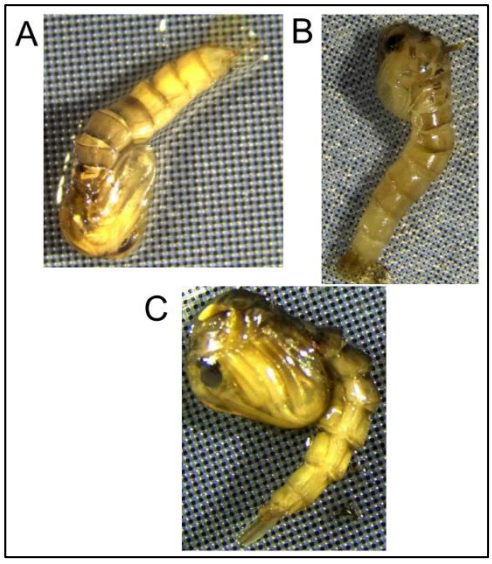


**Figure 4.2:** Examples of mosaic G0 *yellow* pupae. **A** – Y1A, **B** – Y5A, **C** – background strain (Exu-Cs9)

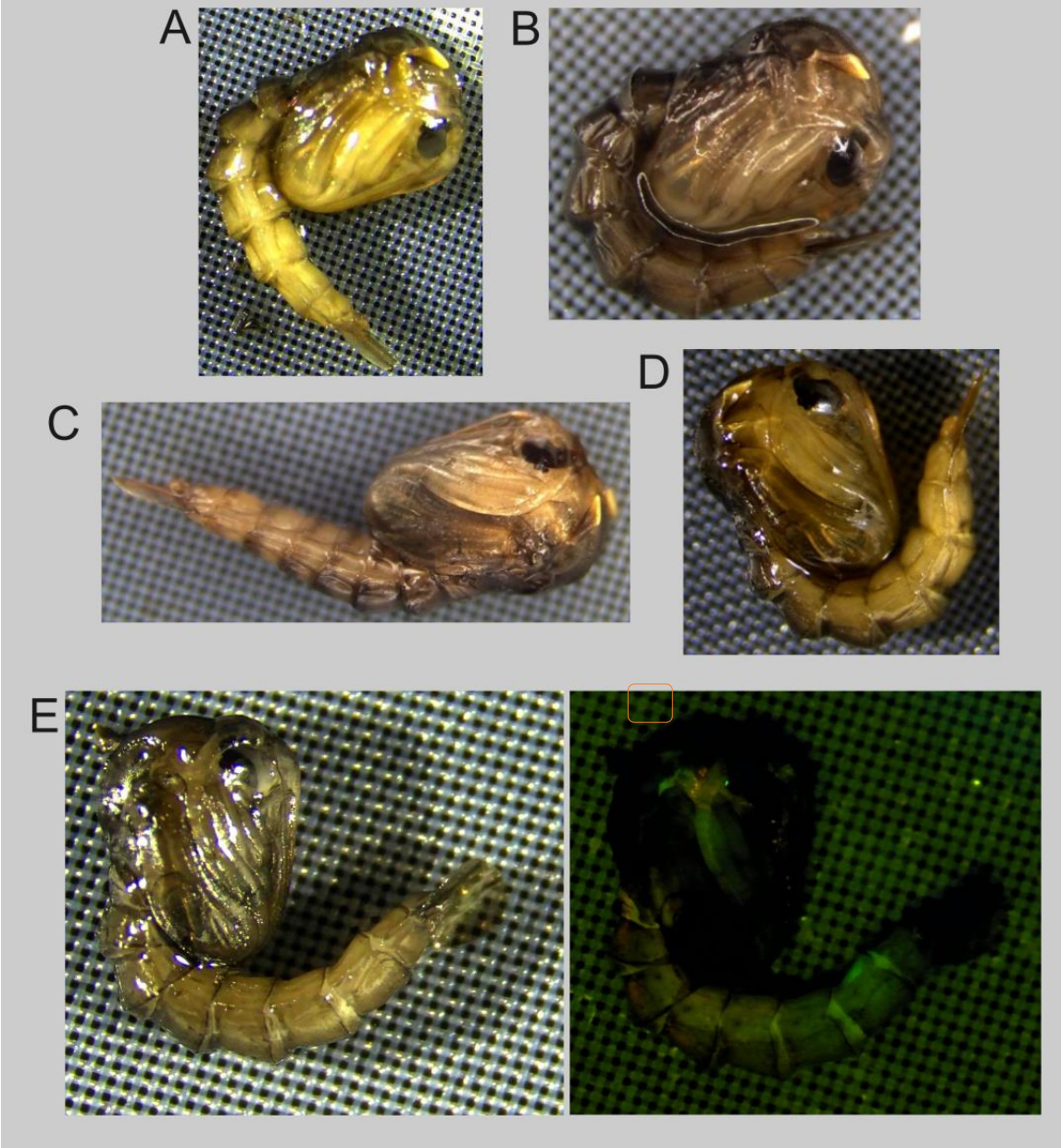


**Figure 4.3:** Examples of mosaic G0 *ebony* pupae. **A** – background strain (Exu-Cas9), **B** – E1B, **C** – E5C, **D**– E5A, **E** – E5B. Only one pupa was positive for GFP in the eye. Figure E shows the positive pupa both in brightfield (left panel) and under the GFP filter (right panel). The GFP fluorescence is highlighted by the rectangle around the glowing part of the eye.

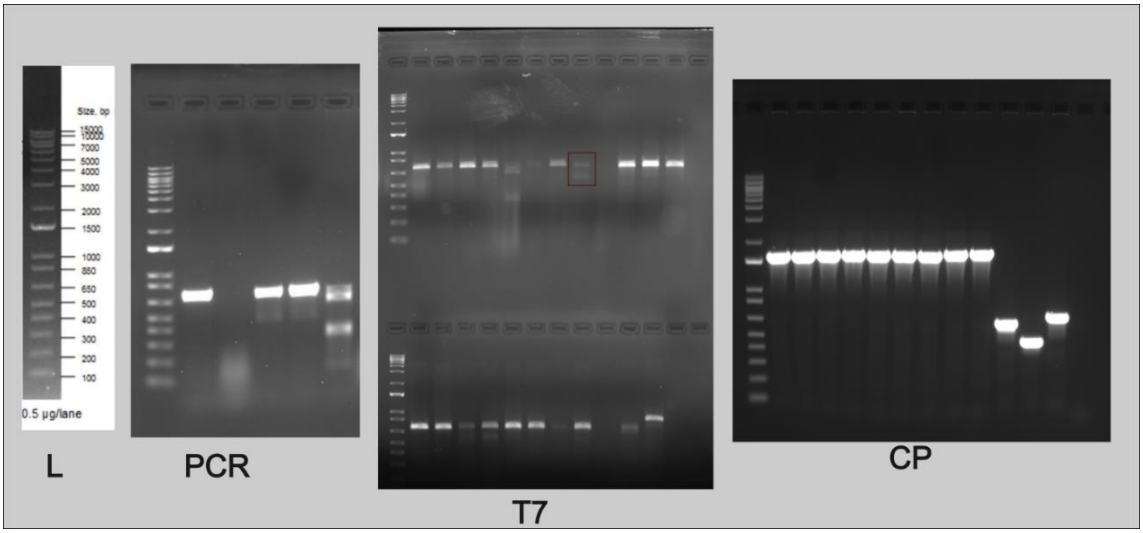


**Figure 4.4:** Molecular evidence of cleavage at the target site in the Ebony-CRISPaint experiment. The ladder L (1 Kb Plus DNA Ladder, Thermo Fisher Scientific Inc., Waltham, MA) is included for reference. The expected size of the amplicon is 717 bp as shown in PCR, an image of the 1% agarose gel electrophoresis of the PCR amplicon. The T7E1 assay products, shown in T7, are separated on a 2% agarose gel to visualize the multiple bands resulting from gene editing. CP is an image of the colony PCR run on a 1% agarose gel after cloning of the PCR amplicon highlighted in red on the T7 image, followed by transformation. The last 3 lanes in CP demonstrate some of the mutant alleles in present in a single pool of two mosquitoes.

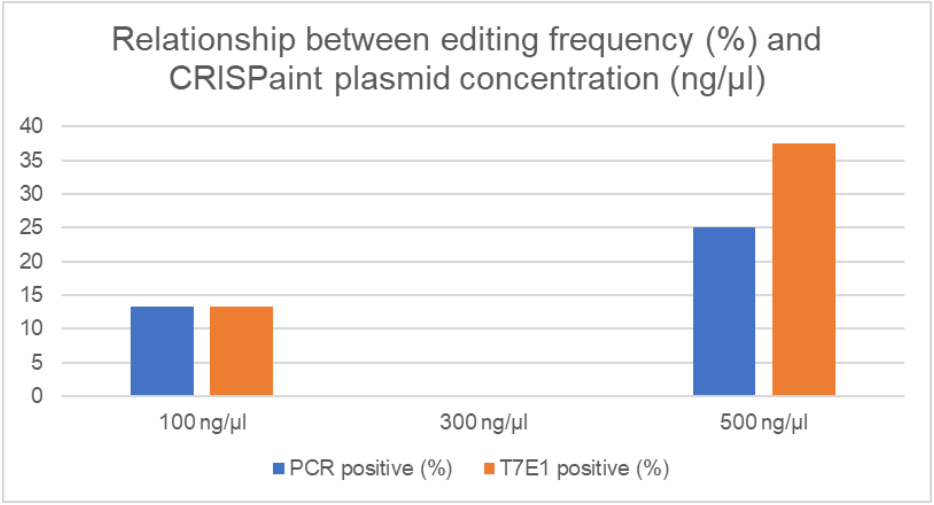


**Figure 4.5:** Graph showing the relationship between the gene editing frequency and the CRISPaint plasmid concentration in the Ebony-CRISPaint experiment.

# CHAPTER FIVE

## CONCLUSION

There is an urgent need for new vector control tools to supplement existing ones. In a world that is rapidly warming, the spread of mosquitoes along with the diseases they vector to new geographical locales that was forecasted earlier (Messina, Brady et al. 2019, Rose, Sylla et al. 2020) come to pass with the first confirmed cases of local malaria transmission in the US in 20 years occurring in June 2023 in Florida. Additional outbreaks of Dengue and Chikungunya in the Americas, as well as the largest West Nile virus outbreak in US history in 2022 further stress the importance of global commitment to mosquito control (Holcomb 2022, Who 2023). To aid this endeavor, the vector control field needs to expand their understanding of vector biology and mosquito-virus interactions.

Viral piRNAs derived from an endogenized form of the virus are a heritable form of immune memory in *Ae. aegypti*, which functions in addition to the siRNA pathway to limit the replication of viruses in the mosquito. While the antiviral activity was only confirmed in the ovaries (Suzuki, Baidaliuk et al. 2020), there is no evidence to suggest that this protection does not extend to somatic tissues. This therefore represents an attractive pathway that can potentially be manipulated to reduce the replication of arboviruses in the mosquito, limiting the transmission of the virus with the next bloodmeal (Fig.5.1).

While the experiments in Chapter 2 of this thesis did not result in piwi knock-out transgenic lines, one way forward would be to attempt tissue specific deletion of the genes to interrogate their functions. This can be performed using the CRISPaint technology to knock-in a plasmid expressing a foldback sequence driven by a midgut-specific promoter such as the *carboxypeptidase A* gene promoter which drives expression in the midgut following a bloodmeal (Franz, Sanchez-Vargas et al. 2006). Other potential promoters, such as *adult peritrophic matrix protein 1* promoter which is active in the midgut independent of a blood meal, have been identified in Anopheline mosquitoes and would need to be validated for use in *Ae. aegypti* (Abraham, Donnelly-Doman et al. 2005).

The decision of which Piwi to target can be informed by tissue-specific co-immunoprecipitation experiments in *Ae. aegypti* itself, not in cell culture. In Chapter 2, blotting for Piwi proteins uncovered the absence of Piwi6 in larvae, which was missed by RNA-seq experiments (Akbari, Antoshechkin et al. 2013). The co-immunoprecipitation experiment may not necessarily point to a ping-pong amplification partner as comparison of small RNA sequencing of somatic and gonadal tissues revealed that the A10 ping-pong signature was only present in germline clusters, suggesting that the somatic piRNAs are derived mainly *via* the phased biogenesis pathway (Gainetdinov, Colpan et al. 2018, Crava, Varghese et al. 2021). Majority of the piRNAs expressed in somatic tissues (adult female carcasses) map to EVEs while in ovaries, majority map to transposable elements (Crava,

Varghese et al. 2021) perhaps reflecting a functional specialization of the pathway in *Ae. aegypti* towards immune memory and protection in the soma.

Midgut-specific *piwi* deletion mosquito strains would allow for a better picture of the involvement of the piRNA pathway and Piwi proteins in mosquito-virus interactions. This would especially be interesting as a study in *Ae. aegypti* revealed that Dengue virus (DENV) is not silenced by siRNAs in adult female midgut after an infected bloodmeal, though the siRNA pathway is active and functional in limiting DENV replication once DENV escapes the midgut and enters into systemic circulation (Olmo, Ferreira et al. 2018). This is surprising as the siRNA pathway is the main form of antiviral immunity in insects and raises the question as to what forms the first line of defense against ingested viruses in the midgut. Relaxed targeting rules exhibited by TE-piRNAs (Gainetdinov, Vega-Badillo et al. 2023) may allow the integrated EVEs to be effective templates for vpiRNAs even after suffering some genetic drift.

Blotting for Piwi4 and Piwi5 in whole larvae and larval midguts uncovered that the proteins are present in sizes a lot smaller than expected. This may reflect enzymatic cleavage that was not suppressed by high protease inhibitor concentrations. Interestingly, in the Piwi5 blots, the protein is absent in whole larvae but present in the larval midgut, suggesting that it is expressed only or mostly in the midgut. Mass spectrometry analysis of the gel between 15 kDa and 25 kDa confirmed that the band obtained in the Western blot is indeed Piwi4. This

raises the question as to whether there is a peptide cleavage site that is exposed in larvae but not in adults which express the full-length protein, or if the cleavage could potentially occur co-transcriptionally. I would answer this by first performing RT-PCR on the larval tissues to determine if the full-length transcript can be obtained. This would be followed by ribosome profiling where mRNA is digested and deep sequencing of ribosome-protected transcripts reveals information on which transcripts are actively being translated, as well as the precise position of the ribosome on the transcript (Ingolia, Brar et al. 2012). The ribosome footprint data may allow for the discrimination between protein enzymatic cleavage and co-translational truncation of Piwi4 and Piwi5 proteins.

Typically, Ago3 is expected to partner with one of the Piwi proteins for ping-pong biogenesis of piRNAs to occur. Immunoblotting for the Piwi proteins in the larval stage of development painted an interesting picture of the pathway in this juvenile stage of development. Piwi2 was not detected in larvae, which was expected as RNA-seq experiments showed that the transcripts were expressed in the blood-fed ovaries and developing embryos. Piwi6 was also not detected in the larval stage though it was expected to be present. Piwi4 and Piwi5 were detected in truncated forms of approximately 20 kDa for Piwi4 and 35 KDa for Piwi5. Mass spectrometry analysis of the gel at that size band confirmed that peptides from Piwi4 are indeed present, ruling out non-specific or off-target binding of the antibody.

This presents an interesting scenario in larvae as the ping-pong biogenesis pathway is not present, therefore mobile genetic elements appear to be handled by primary biogenesis and association with Piwi4 and Piwi5, if these proteins are biologically active. In the ovaries, all tested Piwi sub-family proteins were present as expected, however Ago3 was not detected in the testes. This raises the possibility that the ping-pong amplification pathway may be superfluous for the restriction of the activity of transposable elements in the male germline. This requires further study with a focus on the testes.

Altogether these data lead me to speculate that Ago3 is restricted to the ovaries, and perhaps the developing embryo, where the protein participates in ping-pong amplification of piRNAs directed both at vertically transmitted viruses and TEs. This secondary piRNA biogenesis pathway serves to increase the pool of piRNAs available to protect the integrity of the genome in the germline, thus ensuring the survival of future generations. In the adult testes and in the larval stage of development, viruses and TEs are restricted by the activity of piRNAs produced by phased biogenesis and complexed with either Piwi2 (testes only), Piwi4 or Piwi5.

A recent examination of piRNA expression in *Ae. aegypti* midguts confirmed the choice of piRNA cluster in Chapter 3 experiments, as it was found to be the midgut cluster with the highest expression (Qu, Betting et al. 2023). The same study revealed that piRNA clusters are transcribed by read-through transcription from highly conserved upstream genes (Qu,

Betting et al. 2023). With this knowledge, an artificial endogenous viral element (EVE) can be inserted immediately downstream of the gene. With more injections, and a more conserved target site, a line could be established, and small RNA sequencing done every generation to determine if the artificial EVE expresses piRNAs. The results of this experiment would provide information on how piRNA clusters are formed and confirm the hypothesis that the piRNA pathway functions to provide heritable immune protection in somatic tissues such as the midgut to circulating insect-specific viruses.

*Ae. aegypti* mosquitoes have the remarkable capability to quickly adapt to changing climatic conditions such as drought (Venkataraman, Shai et al. 2023), further underlying the need for a diversity in the tools used to control the arboviral vectors. This is hampered by considerable public opposition to genetically modified mosquitoes, both in California and globally (Thizy, Pare Toe et al. 2021, Schairer, Triplett et al. 2022). Public health education and community engagement are necessary to obtain consent to releases of transgenic mosquitoes in the community, while the development of coherent global policies regarding genetically modified mosquito release programs would go a long way in changing the public's attitude toward transgenic mosquitoes.

BIBLIOGRAPHY

Abraham, E. G., M. Donnelly-Doman, H. Fujioka, A. Ghosh, L. Moreira and M. Jacobs-Lorena (2005). "Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements." Insect Mol. Biol. **14**(3): 271-279.

Adams, K. L., D. G. Abernathy, B. C. Willett, E. K. Selland, M. A. Itoe and F. Catteruccia (2021).

"Wolbachia cifB induces cytoplasmic incompatibility in the malaria mosquito vector." Nat Microbiol **6**(12): 1575-1582.

Adelman, Z. N., I. Sanchez-Vargas, E. A. Travanty, J. O. Carlson, B. J. Beaty, C. D. Blair and K. E. Olson (2002). "RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome." J. Virol. **76**(24): 12925-12933.

Aguiar, E. R. G. R., J. P. P. de Almeida, L. R. Queiroz, L. S. Oliveira, R. P. Olmo, I. J. d. S. de Faria, J.-L. Imler, A. Gruber, B. J. Matthews and J. T. Marques (2020). "A single unidirectional piRNA cluster similar to the flamenco locus is the major source of EVE-derived transcription and small RNAs in Aedes aegypti mosquitoes." RNA **26**(5): 581-594.

Akbari, O. S., I. Antoshechkin, H. Amrhein, B. Williams, R. Diloreto, J. Sandler and B. A. Hay (2013). "The developmental transcriptome of the mosquito Aedes aegypti, an invasive species and major arbovirus vector." G3 **3**(9): 1493-1509.

Amos, B., C. Aurrecoechea, M. Barba, A. Barreto, E. Y. Basenko, W. Bażant, R. Belnap, A. S. Blevins, U. Böhme, J. Brestelli, B. P. Brunk, M. Caddick, D. Callan, L. Campbell, M. B. Christensen, G. K. Christophides, K. Crouch, K. Davis, J. DeBarry, R. Doherty, Y. Duan, M. Dunn, D. Falke, S. Fisher, P. Flicek, B. Fox, B. Gajria, G. I. Giraldo-Calderón, O. S. Harb, E. Harper, C. Hertz-Fowler, M. J. Hickman, C. Howington, S. Hu, J. Humphrey, J. Iodice, A. Jones, J. Judkins, S. A. Kelly, J. C. Kissinger, D. K. Kwon, K. Lamoureux, D. Lawson, W. Li, K. Lies, D. Lodha, J. Long, R. M. MacCallum, G. Maslen, M. A. McDowell, J. Nabrzyski, D. S. Roos, S. S. C. Rund, S. W. Schulman, A. Shanmugasundram, V. Sitnik, D. Spruill, D. Starns, C. J. Stoeckert, S. S. Tomko, H. Wang, S. Warrenfeltz, R. Wieck, P. A. Wilkinson, L. Xu and J. Zheng (2022). "VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center." Nucleic Acids Res. **50**(D1): D898-D911.
Andersen, P. R., L. Tirian, M. Vunjak and J. Brennecke (2017). "A heterochromatin-dependent transcription machinery drives piRNA expression." Nature **549**(7670): 54-59.

Ang, J. X. D., K. Nevard, R. Ireland, D.-K. Purusothaman, S. A. N. Verkuijl, L. Shackleford, E. Gonzalez, M. A. E. Anderson and L. Alphey (2022). "Considerations for homology-based DNA repair in mosquitoes: Impact of sequence heterology and donor template source." PLoS Genet. **18**(2): e1010060.

Ant, T. H., C. S. Herd, V. Geoghegan, A. A. Hoffmann and S. P. Sinkins (2018). "The Wolbachia strain wAu provides highly efficient virus transmission blocking in Aedes aegypti." PLoS Pathog. **14**(1): e1006815.

Ant, T. H. and S. P. Sinkins (2018). "A Wolbachia triple-strain infection generates self-incompatibility in Aedes albopictus and transmission instability in Aedes aegypti." Parasit. Vectors **11**(1): 295.

Aravin, A., D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf, N. Iovino, P. Morris, M. J. Brownstein, S. Kuramochi-Miyagawa, T. Nakano, M. Chien, J. J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan and T. Tuschl (2006). "A novel class of small RNAs bind to MILI protein in mouse testes." Nature **442**(7099): 203-207.

Aravin, A. A., M. Lagos-Quintana, A. Yalcin, M. Zavolan, D. Marks, B. Snyder, T. Gaasterland, J. Meyer and T. Tuschl (2003). "The small RNA profile during Drosophila melanogaster development." Dev. Cell **5**(2): 337-350.

Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky and V. A. Gvozdev (2001). "Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline." Curr. Biol. **11**(13): 1017-1027.

Arensburger, P., R. H. Hice, J. A. Wright, N. L. Craig and P. W. Atkinson (2011). "The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs." BMC Genomics **12**: 606.

Aryan, A., M. A. E. Anderson, K. M. Myles and Z. N. Adelman (2013). "TALEN-based gene disruption in the dengue vector Aedes aegypti." PLoS One **8**(3): e60082.

Asgari, S. (2014). "Role of microRNAs in arbovirus/vector interactions." Viruses **6**(9): 3514-3534.
Atwood, K. C., R. C. Von Borstel and A. R. Whiting (1956). "An Influence of Ploidy on the Time of Expression of Dominant Lethal Mutations in Habrobracon." Genetics **41**(6): 804-813.

Ayettey, J., A. Ablorde, G. K. Amlalo, B. A. Mensah and A. A. Kudom (2023). "Entomological surveillance on Aedes aegypti during covid 19 period in Cape Coast, Ghana: Risk of arboviral outbreaks, multiple insecticide resistance and distribution of F1534C, V410L and V1016I kdr mutations." PLoS Negl. Trop. Dis. **17**(5): e0011397.

Balaji, S., S. Jayachandran and S. R. Prabagaran (2019). "Evidence for the natural occurrence of Wolbachia in Aedes aegypti mosquitoes." FEMS Microbiol. Lett. **366**(6).

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Basu, S., A. Aryan, J. M. Overcash, G. H. Samuel, M. A. E. Anderson, T. J. Dahlem, K. M. Myles and Z. N. Adelman (2015). "Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **112**(13): 4038-4043.
Beckmann, J. F., J. A. Ronau and M. Hochstrasser (2017). "A Wolbachia deubiquitylating enzyme induces cytoplasmic incompatibility." Nat Microbiol **2**: 17007.

Besson, B., O. M. Lezcano, G. J. Overheul, K. Janssen, C. G. Spruijt, M. Vermeulen, J. Qu and R. P. van Rij (2022). "Arbovirus-vector protein interactomics identifies Loquacious as a co-factor for dengue virus replication in Aedes mosquitoes." bioRxiv.

Blair, C. D. and K. E. Olson (2015). "The role of RNA interference (RNAi) in arbovirus-vector interactions." Viruses **7**(2): 820-843.

Blair, C. D., K. E. Olson and M. Bonizzoni (2019). "The Widespread Occurrence and Potential Biological Roles of Endogenous Viral Elements in Insect Genomes." Curr. Issues Mol. Biol. **34**: 13-30.

Bonneau, M., C. Atyame, M. Beji, F. Justy, M. Cohen-Gonsaud, M. Sicard and M. Weill (2018). "Culex pipiens crossing type diversity is governed by an amplified and polymorphic operon of Wolbachia." Nat. Commun. **9**(1): 319.

Bonning, B. C. and M.-C. Saleh (2021). "The Interplay Between Viruses and RNAi Pathways in Insects." Annu. Rev. Entomol. **66**: 61-79.

Bořkovec, A. B. (1976). "Control and Management of Insect Populations by Chemosterilants." Environ. Health Perspect. **14**: 103-107.

Bosch, J. A., R. Colbeth, J. Zirin and N. Perrimon (2019). "Gene knock-ins in Drosophila using homology-independent insertion of universal donor plasmids." bioRxiv.

Bosch, J. A., S. Knight, O. Kanca, J. Zirin, D. Yang-Zhou, Y. Hu, J. Rodiger, G. Amador, H. J. Bellen, N. Perrimon and S. E. Mohr (2020). "Use of the CRISPR-Cas9 System in Drosophila Cultured Cells to Introduce Fluorescent Tags into Endogenous Genes." Curr. Protoc. Mol. Biol. **130**(1): e112.

Bottino-Rojas, V. and A. A. James (2022). "Use of Insect Promoters in Genetic Engineering to Control Mosquito-Borne Diseases." Biomolecules **13**(1).

Brackney, D. E., J. C. Scott, F. Sagawa, J. E. Woodward, N. A. Miller, F. D. Schilkey, J. Mudge, J. Wilusz, K. E. Olson, C. D. Blair and G. D. Ebel (2010). "C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response." PLoS Negl. Trop. Dis. **4**(10): e856.

Breeland, S. G., G. M. Jeffery, C. S. Lofgren and D. E. Weidhaas (1974). "Release of chemosterilized males for the control of Anopheles albimanus in El Salvador. I. Characteristics of the test site and the natural population." Am. J. Trop. Med. Hyg. **23**(2): 274-281.

Brennecke, J., A. A. Aravin, A. Stark, M. Dus, M. Kellis, R. Sachidanandam and G. J. Hannon (2007). "Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila." Cell **128**(6): 1089-1103.

Bushland, R. C. and D. E. Hopkins (1951). "Experiments with Screw-Worm Flies Sterilized by X-Rays." J. Econ. Entomol. **44**(5): 725-731.

Cabrera, M. and K. Jaffe (2007). "An aggregation pheromone modulates lekking behavior in the vector mosquito Aedes aegypti (Diptera: Culicidae)." J. Am. Mosq. Control Assoc. **23**(1): 1-10.

Cammarata, G., D. de Miguel-Perez, A. Russo, A. Peleg, V. Dolo, C. Rolfo and S. Taverna (2022). "Emerging noncoding RNAs contained in extracellular vesicles: rising stars as biomarkers in lung cancer liquid biopsy." Ther. Adv. Med. Oncol. **14**: 17588359221131229.

Campbell, C. L., W. C. t. Black, A. M. Hess and B. D. Foy (2008). "Comparative genomics of small RNA regulatory pathway components in vector mosquitoes." BMC Genomics **9**: 425.

Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." Cell **136**(4): 642-655.

Carvalho, D. O., A. R. McKemey, L. Garziera, R. Lacroix, C. A. Donnelly, L. Alphey, A. Malavasi and M. L. Capurro (2015). "Suppression of a Field Population of Aedes aegypti in Brazil by Sustained Release of Transgenic Male Mosquitoes." PLoS Negl. Trop. Dis. **9**(7): e0003864.

Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, C. Savakis, F. C. Kafatos and A. Crisanti (2000). "Stable germline transformation of the malaria mosquito Anopheles stephensi." Nature **405**(6789): 959-962.

CDC (2022). "Life Cycle of Aedes aegypti and Ae. albopictus Mosquitoes."
Ceccaldi, R., B. Rondinelli and A. D. D'Andrea (2016). "Repair Pathway Choices and Consequences at the Double-Strand Break." Trends Cell Biol. **26**(1): 52-64.

Cerutti, L., N. Mian and A. Bateman (2000). "Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain." Trends Biochem. Sci. **25**(10): 481-482.

Champer, J., R. Reeves, S. Y. Oh, C. Liu, J. Liu, A. G. Clark and P. W. Messer (2017). "Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations." PLoS Genet. **13**(7): e1006796.

Chapados, B. R., Q. Chai, D. J. Hosfield, J. Qiu, B. Shen and J. A. Tainer (2001). "Structural biochemistry of a type 2 RNase H: RNA primer recognition and removal during DNA replication." J. Mol. Biol. **307**(2): 541-556.

Chaverra-Rodriguez, D., V. M. Macias, G. L. Hughes, S. Pujhari, Y. Suzuki, D. R. Peterson, D. Kim, S. McKeand and J. L. Rasgon (2018). "Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing." Nat. Commun. **9**(1): 3008.

Chiruvella, K. K., Z. Liang and T. E. Wilson (2013). "Repair of double-strand breaks by end joining." Cold Spring Harb. Perspect. Biol. **5**(5): a012757.

Christophers, S. R. (1960). "Aedes aegypti (L.) the yellow fever mosquito: its life history, bionomics and structure." Aëdes aegypti (L. ) the Yellow Fever Mosquito: its Life History, Bionomics and Structure.

Clemments, A. N. (2000). The Biology of Mosquitoes Vol 1. New York, CABI.

Coates, C. J., N. Jasinskiene, L. Miyashiro and A. A. James (1998). "Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3748-3751.

Conant, D., T. Hsiau, N. Rossi, J. Oki, T. Maures, K. Waite, J. Yang, S. Joshi, R. Kelso, K. Holden, B. L. Enzmann and R. Stoner (2022). "Inference of CRISPR Edits from Sanger Trace Data." CRISPR J **5**(1): 123-130.

Crava, C. M., F. S. Varghese, E. Pischedda, R. Halbach, U. Palatini, M. Marconcini, L. Gasmi, S. Redmond, Y. Afrane, D. Ayala, C. Paupy, R. Carballar-Lejarazu, P. Miesen, R. P. van Rij and M. Bonizzoni (2021). "Population genomics in the arboviral vector Aedes aegypti reveals the genomic architecture and evolution of endogenous viral elements." Mol. Ecol. **30**(7): 1594-1611.

Crawford, J. E., J. M. Alves, W. J. Palmer, J. P. Day, M. Sylla, R. Ramasamy, S. N. Surendran, W. C. t. Black, A. Pain and F. M. Jiggins (2017). "Population genomics reveals that an anthropophilic population of Aedes aegypti mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector." BMC Biol. **15**(1): 16.

Crawford, J. E., D. W. Clarke, V. Criswell, M. Desnoyer, D. Cornel, B. Deegan, K. Gong, K. C. Hopkins, P. Howell, J. S. Hyde, J. Livni, C. Behling, R. Benza, W. Chen, K. L. Dobson, C. Eldershaw, D. Greeley, Y. Han, B. Hughes, E. Kakani, J. Karbowski, A. Kitchell, E. Lee, T. Lin, J. Liu, M. Lozano, W. MacDonald, J. W. Mains, M. Metlitz, S. N. Mitchell, D. Moore, J. R. Ohm, K. Parkes, A. Porshnikoff, C. Robuck, M. Sheridan, R. Sobecki, P. Smith, J. Stevenson, J. Sullivan, B. Wasson, A. M. Weakley, M. Wilhelm, J. Won, A. Yasunaga, W. C. Chan, J. Holeman, N. Snoad, L. Upson, T. Zha, S. L. Dobson, F. S. Mulligan, P. Massaro and B. J. White (2020). "Efficient production of male Wolbachia-infected Aedes aegypti mosquitoes enables large-scale suppression of wild populations." Nat. Biotechnol. **38**(4): 482-492.

Danet, L., G. Beauclair, M. Berthet, G. Moratorio, S. Gracias, F. Tangy, V. Choumet and N. Jouvenet (2019). "Midgut barriers prevent the replication and dissemination of the yellow fever vaccine in Aedes aegypti." PLoS Negl. Trop. Dis. **13**(8): e0007299.

Darricarrère, N., N. Liu, T. Watanabe and H. Lin (2013). "Function of Piwi, a nuclear Piwi/Argonaute protein, is independent of its slicer activity." Proc. Natl. Acad. Sci. U. S. A. **110**(4): 1297-1302.

de Almeida, J. P., E. R. Aguiar, J. N. Armache, R. P. Olmo and J. T. Marques (2021). "The virome of vector mosquitoes." Curr. Opin. Virol. **49**: 7-12.

Deehan, M., W. Lin, B. Blum, A. Emili and H. Frydman (2021). "Intracellular Density of Wolbachia Is Mediated by Host Autophagy and the Bacterial Cytoplasmic Incompatibility Gene cifB in a Cell Type-Dependent Manner in Drosophila melanogaster." MBio **12**(1).

Degner, E. C. and L. C. Harrington (2016). "Polyandry Depends on Postmating Time Interval in the Dengue Vector Aedes aegypti." Am. J. Trop. Med. Hyg. **94**(4): 780-785.

Deredec, A., A. Burt and H. C. J. Godfray (2008). "The population genetics of using homing endonuclease genes in vector and pest management." Genetics **179**(4): 2013-2026.

Derryberry, O. M. and F. E. Gartrell (1952). "Trends in malaria control program of the Tennessee Valley Authority." Am. J. Trop. Med. Hyg. **1**(3): 500-507.

Dietrich, I., S. Jansen, G. Fall, S. Lorenzen, M. Rudolf, K. Huber, A. Heitmann, S. Schicht, E. H. Ndiaye, M. Watson, I. Castelli, B. Brennan, R. M. Elliott, M. Diallo, A. A. Sall, A.-B. Failloux, E. Schnettler, A. Kohl and S. C. Becker (2017). "RNA Interference Restricts Rift Valley Fever Virus in Multiple Insect Systems." mSphere **2**(3).

Dong, S., J. Lin, N. L. Held, R. J. Clem, A. L. Passarelli and A. W. E. Franz (2015). "Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti." PLoS One **10**(3): e0122353.

Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science **346**(6213): 1258096.

Drinnenberg, I. A., D. E. Weinberg, K. T. Xie, J. P. Mower, K. H. Wolfe, G. R. Fink and D. P. Bartel (2009). "RNAi in budding yeast." Science **326**(5952): 544-550.

El Chamy, L., V. Leclerc, I. Caldelari and J.-M. Reichhart (2008). "Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll." Nat. Immunol. **9**(10): 1165-1170.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes Dev. **15**(2): 188-200.

Elissa, N., J. Mouchet, F. Riviere, J. Y. Meunier and K. Yao (1993). "Resistance of Anopheles gambiae s.s. to pyrethroids in Côte d'Ivoire." Ann. Soc. Belg. Med. Trop. **73**(4): 291-294.

Evans, B. R., P. Kotsakiozi, A. L. Costa-da-Silva, R. S. Ioshino, L. Garziera, M. C. Pedrosa, A. Malavasi, J. F. Virginio, M. L. Capurro and J. R. Powell (2019). "Transgenic Aedes aegypti Mosquitoes Transfer Genes into a Natural Population." Sci. Rep. **9**(1): 13047.

Fawaz, E. Y., S. A. Allan, U. R. Bernier, P. J. Obenauer and J. W. Diclaro, 2nd (2014). "Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of Aedes aegypti." J. Vector Ecol. **39**(2): 347-354.

Ferguson, N. M., D. T. H. Kien, H. Clapham, R. Aguas, V. T. Trung, T. N. B. Chau, J. Popovici, P. A. Ryan, S. L. O'Neill, E. A. McGraw, V. T. Long, L. T. Dui, H. L. Nguyen, N. V. V. Chau, B. Wills and C. P. Simmons (2015). "Modeling the impact on virus transmission of Wolbachia-mediated blocking of dengue virus infection of Aedes aegypti." Sci. Transl. Med. **7**(279): 279ra237.

Ferroni, E., T. Jefferson and G. Gachelin (2012). "Angelo Celli and research on the prevention of malaria in Italy a century ago." J. R. Soc. Med. **105**(1): 35-40.

Frank, F., N. Sonenberg and B. Nagar (2010). "Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2." Nature **465**(7299): 818-822.

Frank, J. A. and C. Feschotte (2017). "Co-option of endogenous viral sequences for host cell function." Curr. Opin. Virol. **25**: 81-89.

Franz, A. W. E., A. M. Kantor, A. L. Passarelli and R. J. Clem (2015). "Tissue Barriers to Arbovirus Infection in Mosquitoes." Viruses **7**(7): 3741-3767.

Franz, A. W. E., I. Sanchez-Vargas, Z. N. Adelman, C. D. Blair, B. J. Beaty, A. A. James and K. E. Olson (2006). "Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **103**(11): 4198-4203.

Fredericks, A. C., T. A. Russell, L. E. Wallace, A. D. Davidson, A. Fernandez-Sesma and K. Maringer (2019). "Aedes aegypti (Aag2)-derived clonal mosquito cell lines reveal the effects of pre-existing persistent infection with the insect-specific bunyavirus Phasi Charoen-like virus on arbovirus replication." PLoS Negl. Trop. Dis. **13**(11): e0007346.

Gainetdinov, I., C. Colpan, A. Arif, K. Cecchini and P. D. Zamore (2018). "A Single Mechanism of Biogenesis, Initiated and Directed by PIWI Proteins, Explains piRNA Production in Most Animals." Mol. Cell **71**(5): 775-790.e775.

Gainetdinov, I., J. Vega-Badillo, K. Cecchini, A. Bagci, C. Colpan, D. De, S. Bailey, A. Arif, P.-H. Wu, I. J. MacRae and P. D. Zamore (2023). "Relaxed targeting rules help PIWI proteins silence transposons." Nature.

Galton, R., K. Fejes-Toth and M. E. Bronner (2022). "Co-option of the piRNA pathway to regulate neural crest specification." Science Advances **8**(32): eabn1441.

Gan, S. J., Y. Q. Leong, M. F. H. Bin Barhanuddin, S. T. Wong, S. F. Wong, J. W. Mak and R. B. Ahmad (2021). "Dengue fever and insecticide resistance in Aedes mosquitoes in Southeast Asia: a review." Parasit. Vectors **14**(1): 315.

Gantz, V. M., N. Jasinskiene, O. Tatarenkova, A. Fazekas, V. M. Macias, E. Bier and A. A. James (2015). "Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi." Proc. Natl. Acad. Sci. U. S. A. **112**(49): E6736-6743.

Geoghegan, V., K. Stainton, S. M. Rainey, T. H. Ant, A. A. Dowle, T. Larson, S. Hester, P. D. Charles, B. Thomas and S. P. Sinkins (2017). "Perturbed cholesterol and vesicular trafficking associated with dengue blocking in Wolbachia-infected Aedes aegypti cells." Nat. Commun. **8**(1): 526.

Girardi, E., P. Miesen, B. Pennings, L. Frangeul, M.-C. Saleh and R. P. van Rij (2017). "Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in Aedes aegypti." Nucleic Acids Res. **45**(8): 4881-4892.

Gloria-Soria, A., T. G. Chiodo and J. R. Powell (2018). "Lack of Evidence for Natural Wolbachia Infections in Aedes aegypti (Diptera: Culicidae)." J. Med. Entomol. **55**(5): 1354-1356.

Göertz, G. P., J. J. Fros, P. Miesen, C. B. F. Vogels, M. L. van der Bent, C. Geertsema, C. J. M. Koenraadt, R. P. van Rij, M. M. van Oers and G. P. Pijlman (2016). "Noncoding Subgenomic Flavivirus RNA Is Processed by the Mosquito RNA Interference Machinery and Determines West Nile Virus Transmission by Culex pipiens Mosquitoes." J. Virol. **90**(22): 10145-10159.

Goic, B., K. A. Stapleford, L. Frangeul, A. J. Doucet, V. Gausson, H. Blanc, N. Schemmel-Jofre, G. Cristofari, L. Lambrechts, M. Vignuzzi and M.-C. Saleh (2016). "Virus-derived DNA drives mosquito vector tolerance to arboviral infection." Nat. Commun. **7**: 12410.

Goic, B., N. Vodovar, J. A. Mondotte, C. Monot, L. Frangeul, H. Blanc, V. Gausson, J. Vera-Otarola, G. Cristofari and M.-C. Saleh (2013). "RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila." Nat. Immunol. **14**(4): 396-403.

Goriaux, C., S. Desset, Y. Renaud, C. Vaury and E. Brasset (2014). "Transcriptional properties and splicing of the flamenco piRNA cluster." EMBO Rep. **15**(4): 411-418.

Grossman, G. L., C. S. Rafferty, J. R. Clayton, T. K. Stevens, O. Mukabayire and M. Q. Benedict (2001). "Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element." Insect Mol. Biol. **10**(6): 597-604.

Grubaugh, N. D., J. Weger-Lucarelli, R. A. Murrieta, J. R. Fauver, S. M. Garcia-Luna, A. N. Prasad, W. C. t. Black and G. D. Ebel (2016). "Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching." Cell Host Microbe **19**(4): 481-492.

Gunawardane, L. S., K. Saito, K. M. Nishida, K. Miyoshi, Y. Kawamura, T. Nagami, H. Siomi and M. C. Siomi (2007). "A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila." Science **315**(5818): 1587-1590.

Gwadz, R. W., G. B. Craig, Jr. and W. A. Hickey (1971). "Female sexual behavior as the mechanism rendering Aedes aegypti refractory to insemination." Biol. Bull. **140**(2): 201-214.

Halbach, R., P. Miesen, J. Joosten, E. Taşköprü, I. Rondeel, B. Pennings, C. B. F. Vogels, S. H. Merkling, C. J. Koenraadt, L. Lambrechts and R. P. van Rij (2020). "A satellite repeat-derived piRNA controls embryonic development of Aedes." Nature.

Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, A. Burt, N. Windbichler, A. Crisanti and T. Nolan (2016). "A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae." Nat. Biotechnol. **34**(1): 78-83.

Hammond, A., P. Pollegioni, T. Persampieri, A. North, R. Minuz, A. Trusso, A. Bucci, K. Kyrou, I. Morianou, A. Simoni, T. Nolan, R. Müller and A. Crisanti (2021). "Gene-drive suppression of mosquito populations in large cages as a bridge between lab and field." Nat. Commun. **12**(1): 4589.

Hammond, A. M., K. Kyrou, M. Bruttini, A. North, R. Galizi, X. Karlsson, N. Kranjc, F. M. Carpi, R. D'Aurizio, A. Crisanti and T. Nolan (2017). "The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito." PLoS Genet. **13**(10): e1007039.

Hammond, S. M., E. Bernstein, D. Beach and G. J. Hannon (2000). "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells." Nature **404**(6775): 293-296.

Han, M. E. (2017). The piRNA System in Aedes aegypti. Ph.D. Ph.D. Thesis, University of California, Riverside.

Handler, A. M. (2001). "A current perspective on insect gene transformation." Insect Biochem. Mol. Biol. **31**(2): 111-128.

Harris, A. F., A. R. McKemey, D. Nimmo, Z. Curtis, I. Black, S. A. Morgan, M. N. Oviedo, R. Lacroix, N. Naish, N. I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C. A. Donnelly, W. D. Petrie and L. Alphey (2012).

"Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes." Nat. Biotechnol. **30**(9): 828-830.

Harris, A. F., D. Nimmo, A. R. McKemey, N. Kelly, S. Scaife, C. A. Donnelly, C. Beech, W. D. Petrie and L. Alphey (2011). "Field performance of engineered male mosquitoes." Nat. Biotechnol. **29**(11): 1034-1037.

Hartberg, W. K. (1971). "Observations on the mating behaviour of Aedes aegypti in nature." Bull. World Health Organ. **45**(6): 847-850.

Hayashi, R., J. Schnabl, D. Handler, F. Mohn, S. L. Ameres and J. Brennecke (2016). "Genetic and mechanistic diversity of piRNA 3'-end formation." Nature **539**(7630): 588-592.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill and K. N. Johnson (2008). "Wolbachia and virus protection in insects." Science **322**(5902): 702.

Hess, A. M., A. N. Prasad, A. Ptitsyn, G. D. Ebel, K. E. Olson, C. Barbacioru, C. Monighetti and C. L. Campbell (2011). "Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense." BMC Microbiol. **11**: 45.

Higashitani, Y. and K. Horie (2023). "Long-read sequence analysis of MMEJ-mediated CRISPR genome editing reveals complex on-target vector insertions that may escape standard PCR-based quality control." Sci. Rep. **13**(1): 11652.

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, H. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli and S. L. O'Neill (2011). "Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission." Nature **476**(7361): 454-457.

Holcomb, K. (2022). "Worst-ever U.S. West Nile virus outbreak potentially linked to a wetter-than-average 2021 Southwest monsoon."

Huang, Y., Z. Liu and Y. S. Rong (2016). "Genome Editing: From Drosophila to Non-Model Insects and Beyond." J. Genet. Genomics **43**(5): 263-272.

Hussain, M., S. Torres, E. Schnettler, A. Funk, A. Grundhoff, G. P. Pijlman, A. A. Khromykh and S. Asgari (2012). "West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells." Nucleic Acids Res. **40**(5): 2210-2223.

Hutvagner, G. and M. J. Simard (2008). "Argonaute proteins: key players in RNA silencing." Nat. Rev. Mol. Cell Biol. **9**(1): 22-32.

Ingolia, N. T., G. A. Brar, S. Rouskin, A. M. McGeachy and J. S. Weissman (2012). "The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments." Nat. Protoc. **7**(8): 1534-1550.

Jangir, P. K. and A. Prasad (2022). "Spatial distribution of insecticide resistance and susceptibility in Aedes aegypti and Aedes albopictus in India." Int. J. Trop. Insect Sci. **42**(2): 1019-1044.

Jasinskiene, N., C. J. Coates, M. Q. Benedict, A. J. Cornel, C. S. Rafferty, A. A. James and F. H. Collins (1998). "Stable transformation of the yellow fever mosquito, Aedes aegypti, with the Hermes element from the housefly." Proc. Natl. Acad. Sci. U. S. A. **95**(7): 3743-3747.

Jehn, J., D. Gebert, F. Pipilescu, S. Stern, J. S. T. Kiefer, C. Hewel and D. Rosenkranz (2018). "PIWI genes and piRNAs are ubiquitously expressed in mollusks and show patterns of lineage-specific adaptation." Commun Biol **1**: 137.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science **337**(6096): 816-821.

Joosten, J., P. Miesen, E. Taşköprü, B. Pennings, P. W. T. C. Jansen, M. A. Huynen, M. Vermeulen and R. P. Van Rij (2019). "The Tudor protein Veneno assembles the ping-pong amplification complex that produces viral piRNAs in Aedes mosquitoes." Nucleic Acids Res. **47**(5): 2546-2559.

Kakumani, P. K., S. S. Ponia, R. K. S, V. Sood, M. Chinnappan, A. C. Banerjea, G. R. Medigeshi, P. Malhotra, S. K. Mukherjee and R. K. Bhatnagar (2013). "Role of RNA interference (RNAi) in dengue virus replication and identification of NS4B as an RNAi suppressor." J. Virol. **87**(16): 8870-8883.

Kandul, N. P., J. Liu, J. B. Bennett, J. M. Marshall and O. S. Akbari (2021). "A confinable home-and-rescue gene drive for population modification." Elife **10**.

Kandul, N. P., J. Liu, H. M. Sanchez C, S. L. Wu, J. M. Marshall and O. S. Akbari (2019). "Transforming insect population control with precision guided sterile males with demonstration in flies." Nat. Commun. **10**(1): 84.

Katzourakis, A. and R. J. Gifford (2010). "Endogenous viral elements in animal genomes." PLoS Genet. **6**(11): e1001191.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat. Rev. Mol. Cell Biol. **10**(2): 126-139.

Kistler, K. E., L. B. Vosshall and B. J. Matthews (2015). "Genome engineering with CRISPR-Cas9 in the mosquito Aedes aegypti." Cell Rep. **11**(1): 51-60.

Kittayapong, P. (2021). Combined sterile insect technique and incompatible insect technique: concept, study design, experience and lessons learned from a pilot suppression trial in Thailand. Area-Wide Integrated Pest Management, CRC Press**:** 405-432.

Kittayapong, P., S. Ninphanomchai, W. Limohpasmanee, C. Chansang, U. Chansang and P. Mongkalangoon (2019). "Combined sterile insect technique and incompatible insect technique: The

first proof-of-concept to suppress Aedes aegypti vector populations in semi-rural settings in Thailand." PLoS Negl. Trop. Dis. **13**(10): e0007771.

Kiuchi, T., H. Koga, M. Kawamoto, K. Shoji, H. Sakai, Y. Arai, G. Ishihara, S. Kawaoka, S. Sugano, T. Shimada, Y. Suzuki, M. G. Suzuki and S. Katsuma (2014). "A single female-specific piRNA is the primary determiner of sex in the silkworm." Nature **509**(7502): 633-636.

Klattenhoff, C., H. Xi, C. Li, S. Lee, J. Xu, J. S. Khurana, F. Zhang, N. Schultz, B. S. Koppetsch, A. Nowosielska, H. Seitz, P. D. Zamore, Z. Weng and W. E. Theurkauf (2009). "The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters." Cell **138**(6): 1137-1149.

Knipling, E. F. (1979). The Basic Principles of Insect Population Suppression and Management, U.S. Department of Agriculture.

Kokoza, V., A. Ahmed, E. A. Wimmer and A. S. Raikhel (2001). "Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[3xP3-EGFP afm]." Insect Biochem. Mol. Biol. **31**(12): 1137-1143.

Koopal, B., S. K. Mutte and D. C. Swarts (2023). "A long look at short prokaryotic Argonautes." Trends Cell Biol. **33**(7): 605-618.

Kosicki, M., K. Tomberg and A. Bradley (2018). "Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements." Nat. Biotechnol. **36**(8): 765-771.

Kyrou, K., A. M. Hammond, R. Galizi, N. Kranjc, A. Burt, A. K. Beaghton, T. Nolan and A. Crisanti (2018). "A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes." Nat. Biotechnol. **36**(11): 1062-1066.

Lambrechts, L. and W. Scott Thomas (2009). "Mode of transmission and the evolution of arbovirus virulence in mosquito vectors." Proceedings of the Royal Society B: Biological Sciences **276**(1660): 1369-1378.

Lan, Q. and A. M. Fallon (1990). "Small heat shock proteins distinguish between two mosquito species and confirm identity of their cell lines." Am. J. Trop. Med. Hyg. **43**(6): 669-676.

Laven, H., J. Cousserans and G. Guille (1972). "Eradicating mosquitoes using translocations: a first field experiment." Nature **236**(5348): 456-457.

Le Thomas, A., A. K. Rogers, A. Webster, G. K. Marinov, S. E. Liao, E. M. Perkins, J. K. Hur, A. A. Aravin and K. F. Tóth (2013). "Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state." Genes Dev. **27**(4): 390-399.

Le Thomas, A., E. Stuwe, S. Li, J. Du, G. Marinov, N. Rozhkov, Y.-C. A. Chen, Y. Luo, R. Sachidanandam, K. F. Toth, D. Patel and A. A. Aravin (2014). "Transgenerationally inherited piRNAs trigger piRNA biogenesis by changing the chromatin of piRNA clusters and inducing precursor processing." Genes Dev. **28**(15): 1667-1680.

Lee, Y., H. Schmidt, T. C. Collier, W. R. Conner, M. J. Hanemaaijer, M. Slatkin, J. M. Marshall, J. C. Chiu, C. T. Smartt, G. C. Lanzaro, F. S. Mulligan and A. J. Cornel (2019). "Genome-wide divergence among invasive populations of Aedes aegypti in California." BMC Genomics **20**(1): 204.

Leftwich, P. T., L. G. Spurgin, T. Harvey-Samuel, C. J. E. Thomas, L. C. Paladino, M. P. Edgington and L. Alphey (2021). "Genetic pest management and the background genetics of release strains." Philos. Trans. R. Soc. Lond. B Biol. Sci. **376**(1818): 20190805.

Lemaitre, B. and J. Hoffmann (2007). "The host defense of Drosophila melanogaster." Annu. Rev. Immunol. **25**: 697-743.

LePage, D. P., J. A. Metcalf, S. R. Bordenstein, J. On, J. I. Perlmutter, J. D. Shropshire, E. M. Layton, L. J. Funkhouser-Jones, J. F. Beckmann and S. R. Bordenstein (2017). "Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility." Nature **543**(7644): 243-247.

Lewis, S. H., K. A. Quarles, Y. Yang, M. Tanguy, L. Frézal, S. A. Smith, P. P. Sharma, R. Cordaux, C. Gilbert, I. Giraud, D. H. Collins, P. D. Zamore, E. A. Miska, P. Sarkies and F. M. Jiggins (2018). "Pan-arthropod analysis reveals somatic piRNAs as an ancestral defence against transposable elements." Nat Ecol Evol **2**(1): 174-181.

Lewis, S. H., H. Salmela and D. J. Obbard (2016). "Duplication and Diversification of Dipteran Argonaute Genes, and the Evolutionary Divergence of Piwi and Aubergine." Genome Biol. Evol. **8**(3): 507-518.

Lewis, S. H., C. L. Webster, H. Salmela and D. J. Obbard (2016). "Repeated Duplication of Argonaute2 Is Associated with Strong Selection and Testis Specialization in Drosophila." Genetics.
Li, M., M. Bui, T. Yang, C. S. Bowman, B. J. White and O. S. Akbari (2017). "Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **114**(49): E10540-E10549.

Li, M., T. Yang, M. Bui, S. Gamez, T. Wise, N. P. Kandul, J. Liu, L. Alcantara, H. Lee, J. R. Edula, R. Raban, Y. Zhan, Y. Wang, N. DeBeaubien, J. Chen, H. M. Sánchez C, J. B. Bennett, I. Antoshechkin, C. Montell, J. M. Marshall and O. S. Akbari (2021). "Suppressing mosquito populations with precision guided sterile males." Nat. Commun. **12**(1): 5374.

Li, M., T. Yang, N. P. Kandul, M. Bui, S. Gamez, R. Raban, J. Bennett, H. M. Sánchez C, G. C. Lanzaro, H. Schmidt, Y. Lee, J. M. Marshall and O. S. Akbari (2020). "Development of a confinable gene drive system in the human disease vector Aedes aegypti." Elife **9**.

Li, W.-X., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. A. Brydon, K. L. Johnson, A. García-Sastre, L. A. Ball, P. Palese and S.-W. Ding (2004). "Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing." Proc. Natl. Acad. Sci. U. S. A. **101**(5): 1350-1355.

Li, X. Z., C. K. Roy, X. Dong, E. Bolcun-Filas, J. Wang, B. W. Han, J. Xu, M. J. Moore, J. C. Schimenti, Z. Weng and P. D. Zamore (2013). "An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes." Mol. Cell **50**(1): 67-81.

Lindquist, D. A., M. Abusowa and M. J. Hall (1992). "The New World screwworm fly in Libya: a review of its introduction and eradication." Med. Vet. Entomol. **6**(1): 2-8.

Liu-Helmersson, J., H. Stenlund, A. Wilder-Smith and J. Rocklöv (2014). "Vectorial capacity of Aedes aegypti: effects of temperature and implications for global dengue epidemic potential." PLoS One **9**(3): e89783.

Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J.-J. Song, S. M. Hammond, L. Joshua-Tor and G. J. Hannon (2004). "Argonaute2 is the catalytic engine of mammalian RNAi." Science **305**(5689): 1437-1441.

Liu, Q. and Z. Paroo (2010). "Biochemical principles of small RNA pathways." Annu. Rev. Biochem. **79**: 295-319.

Lobo, N. F., A. Hua-Van, X. Li, B. M. Nolen and M. J. Fraser, Jr. (2002). "Germ line transformation of the yellow fever mosquito, Aedes aegypti, mediated by transpositional insertion of a piggyBac vector." Insect Mol. Biol. **11**(2): 133-139.

Lounibos, L. P. (1981). "Habitat segregation among African treehole mosquitoes." Ecol. Entomol. **6**(2): 129-154.

Lu, H.-L., S. Tanguy, C. Rispe, J.-P. Gauthier, T. Walsh, K. Gordon, O. Edwards, D. Tagu, C.-C. Chang and S. Jaubert-Possamai (2011). "Expansion of Genes Encoding piRNA-Associated Argonaute Proteins in the Pea Aphid: Diversification of Expression Profiles in Different Plastic Morphs." PLoS One **6**(12): e28051.

Lutomiah, J. J. L., C. Mwandawiro, J. Magambo and R. C. Sang (2007). "Infection and vertical transmission of Kamiti river virus in laboratory bred Aedes aegypti mosquitoes." J. Insect Sci. **7**: 1-7.
Ma, J.-B., Y.-R. Yuan, G. Meister, Y. Pei, T. Tuschl and D. J. Patel (2005). "Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein." Nature **434**(7033): 666-670.

Malone, C. D., J. Brennecke, M. Dus, A. Stark, W. R. McCombie, R. Sachidanandam and G. J. Hannon (2009). "Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary." Cell **137**(3): 522-535.

Marconcini, M., L. Hernandez, G. Iovino, V. Houé, F. Valerio, U. Palatini, E. Pischedda, J. E. Crawford, B. J. White, T. Lin, R. Carballar-Lejarazu, L. Ometto, F. Forneris, A.-B. Failloux and M. Bonizzoni (2019). "Polymorphism analyses and protein modelling inform on functional specialization of Piwi clade genes in the arboviral vector Aedes albopictus." PLoS Negl. Trop. Dis. **13**(12): e0007919.

Marconcini, M., E. Pischedda, V. Houé, U. Palatini, N. Lozada-Chávez, D. Sogliani, A.-B. Failloux and M. Bonizzoni (2021). "Profile of Small RNAs, vDNA Forms and Viral Integrations in Late Chikungunya Virus Infection of Aedes albopictus Mosquitoes." Viruses **13**(4): 553.

Martinez, J. and T. Tuschl (2004). "RISC is a 5' phosphomonoester-producing RNA endonuclease." Genes Dev. **18**(9): 975-980.

Matsumoto, N., H. Nishimasu, K. Sakakibara, K. M. Nishida, T. Hirano, R. Ishitani, H. Siomi, M. C. Siomi and O. Nureki (2016). "Crystal Structure of Silkworm PIWI-Clade Argonaute Siwi Bound to piRNA." Cell **167**(2): 484-497.e489.

Matthews, B. J., O. Dudchenko, S. B. Kingan, S. Koren, I. Antoshechkin, J. E. Crawford, W. J. Glassford, M. Herre, S. N. Redmond, N. H. Rose, G. D. Weedall, Y. Wu, S. S. Batra, C. A. Brito-Sierra, S. D. Buckingham, C. L. Campbell, S. Chan, E. Cox, B. R. Evans, T. Fansiri, I. Filipović, A. Fontaine, A. Gloria-Soria, R. Hall, V. S. Joardar, A. K. Jones, R. G. G. Kay, V. K. Kodali, J. Lee, G. J. Lycett, S. N. Mitchell, J. Muehling, M. R. Murphy, A. D. Omer, F. A. Partridge, P. Peluso, A. P. Aiden, V. Ramasamy, G. Rašić, S. Roy, K. Saavedra-Rodriguez, S. Sharan, A. Sharma, M. L. Smith, J. Turner, A. M. Weakley, Z. Zhao, O. S. Akbari, W. C. t. Black, H. Cao, A. C. Darby, C. A. Hill, J. S. Johnston, T. D. Murphy, A. S. Raikhel, D. B. Sattelle, I. V. Sharakhov, B. J. White, L. Zhao, E. L. Aiden, R. S. Mann, L. Lambrechts, J. R. Powell, M. V. Sharakhova, Z. Tu, H. M. Robertson, C. S. McBride, A. R. Hastie, J. Korlach, D. E. Neafsey, A. M. Phillippy and L. B. Vosshall (2018). "Improved reference genome of Aedes aegypti informs arbovirus vector control." Nature **563**(7732): 501-507.

McVey, M. and S. E. Lee (2008). "MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings." Trends Genet. **24**(11): 529-538.

Meister, G. (2013). "Argonaute proteins: functional insights and emerging roles." Nat. Rev. Genet. **14**(7): 447-459.

Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl (2004). "Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs." Mol. Cell **15**(2): 185-197.
Merkling, S. H. and R. P. van Rij (2013). "Beyond RNAi: antiviral defense strategies in Drosophila and mosquito." J. Insect Physiol. **59**(2): 159-170.

Messina, J. P., O. J. Brady, N. Golding, M. U. G. Kraemer, G. R. W. Wint, S. E. Ray, D. M. Pigott, F. M. Shearer, K. Johnson, L. Earl, L. B. Marczak, S. Shirude, N. Davis Weaver, M. Gilbert, R. Velayudhan, P. Jones, T. Jaenisch, T. W. Scott, R. C. Reiner, Jr. and S. I. Hay (2019). "The current and future global distribution and population at risk of dengue." Nat Microbiol **4**(9): 1508-1515.

Metzger, M. E., M. Hardstone Yoshimizu, K. A. Padgett, R. Hu and V. L. Kramer (2017). "Detection and Establishment of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) Mosquitoes in California, 2011-2015." J. Med. Entomol. **54**(3): 533-543.

Miesen, P., E. Girardi and R. P. van Rij (2015). "Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in Aedes aegypti mosquito cells." Nucleic Acids Res. **43**(13): 6545-6556.

Miesen, P., A. Ivens, A. H. Buck and R. P. van Rij (2016). "Small RNA Profiling in Dengue Virus 2-Infected Aedes Mosquito Cells Reveals Viral piRNAs and Novel Host miRNAs." PLoS Negl. Trop. Dis. **10**(2): e0004452.

Miesen, P., J. Joosten and R. P. van Rij (2016). "PIWIs Go Viral: Arbovirus-Derived piRNAs in Vector Mosquitoes." PLoS Pathog. **12**(12): e1006017.

Mohn, F., D. Handler and J. Brennecke (2015). "Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis." Science **348**(6236): 812-817.

Mohn, F., G. Sienski, D. Handler and J. Brennecke (2014). "The rhino-deadlock-cutoff complex licenses noncanonical transcription of dual-strand piRNA clusters in Drosophila." Cell **157**(6): 1364-1379.

Mondotte, J. A., V. Gausson, L. Frangeul, Y. Suzuki, M. Vazeille, V. Mongelli, H. Blanc, A.-B. Failloux and M.-C. Saleh (2020). "Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects." Cell Rep. **33**(11): 108506.

Morazzani, E. M., M. R. Wiley, M. G. Murreddu, Z. N. Adelman and K. M. Myles (2012). "Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma." PLoS Pathog. **8**(1): e1002470.

Moyes, C. L., J. Vontas, A. J. Martins, L. C. Ng, S. Y. Koou, I. Dusfour, K. Raghavendra, J. Pinto, V. Corbel, J.-P. David and D. Weetman (2017). "Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans." PLoS Negl. Trop. Dis. **11**(7): e0005625.

Murzin, A. G. (1993). "OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences." EMBO J. **12**(3): 861-867.

Myles, K. M., D. J. Pierro and K. E. Olson (2003). "Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti." J. Virol. **77**(16): 8872-8881.

Nájera, J. A., M. González-Silva and P. L. Alonso (2011). "Some lessons for the future from the Global Malaria Eradication Programme (1955-1969)." PLoS Med. **8**(1): e1000412.

Nakanishi, K., D. E. Weinberg, D. P. Bartel and D. J. Patel (2012). "Structure of yeast Argonaute with guide RNA." Nature **486**(7403): 368-374.

Navarro-Payá, D., I. Flis, M. A. E. Anderson, P. Hawes, M. Li, O. S. Akbari, S. Basu and L. Alphey (2020). "Targeting female flight for genetic control of mosquitoes." PLoS Negl. Trop. Dis. **14**(12): e0008876.

Ngugi, H. N., F. M. Mutuku, B. A. Ndenga, P. S. Musunzaji, J. O. Mbakaya, P. Aswani, L. W. Irungu, D. Mukoko, J. Vulule, U. Kitron and A. D. LaBeaud (2017). "Characterization and productivity profiles of Aedes aegypti (L.) breeding habitats across rural and urban landscapes in western and coastal Kenya." Parasit. Vectors **10**(1): 331.

Nishida, K. M., K. Saito, T. Mori, Y. Kawamura, T. Nagami-Okada, S. Inagaki, H. Siomi and M. C. Siomi (2007). "Gene silencing mechanisms mediated by Aubergine piRNA complexes in Drosophila male gonad." RNA **13**(11): 1911-1922.

Nosrat, C., J. Altamirano, A. Anyamba, J. M. Caldwell, R. Damoah, F. Mutuku, B. Ndenga and A. D. LaBeaud (2021). "Impact of recent climate extremes on mosquito-borne disease transmission in Kenya." PLoS Negl. Trop. Dis. **15**(3): e0009182.

O'Brochta, D. A. and P. W. Atkinson (1996). "Transposable elements and gene transformation in non-drosophilid insects." Insect Biochem. Mol. Biol. **26**(8-9): 739-753.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin and S. L. Dobson (2012). "Open release of male mosquitoes infected with a wolbachia biopesticide: field performance and infection containment." PLoS Negl. Trop. Dis. **6**(11): e1797.

Olmo, R. P., A. G. A. Ferreira, T. C. Izidoro-Toledo, E. R. G. R. Aguiar, I. J. S. de Faria, K. P. R. de Souza, K. P. Osório, L. Kuhn, P. Hammann, E. G. de Andrade, Y. M. Todjro, M. N. Rocha, T. H. J. F. Leite, S. C. G. Amadou, J. N. Armache, S. Paro, C. D. de Oliveira, F. D. Carvalho, L. A. Moreira, E. Marois, J.-L. Imler and J. T. Marques (2018). "Control of dengue virus in the midgut of Aedes aegypti by ectopic expression of the dsRNA-binding protein Loqs2." Nat Microbiol **3**(12): 1385-1393.

Olmo, R. P., Y. M. H. Todjro, E. R. G. R. Aguiar, J. P. P. de Almeida, F. V. Ferreira, J. N. Armache, I. J. S. de Faria, A. G. A. Ferreira, S. C. G. Amadou, A. T. S. Silva, K. P. R. de Souza, A. P. P. Vilela, A. Babarit, C. H. Tan, M. Diallo, A. Gaye, C. Paupy, J. Obame-Nkoghe, T. M. Visser, C. J. M. Koenraadt, M. A. Wongsokarijo, A. L. C. Cruz, M. T. Prieto, M. C. P. Parra, M. L. Nogueira, V. Avelino-Silva, R. N. Mota, M. A. Z. Borges, B. P. Drumond, E. G. Kroon, M. Recker, L. Sedda, E. Marois, J.-L. Imler and J. T. Marques (2023). "Mosquito vector competence for dengue is modulated by insect-specific viruses." Nat Microbiol **8**(1): 135-149.

Ophinni, Y., U. Palatini, Y. Hayashi and N. F. Parrish (2019). "piRNA-Guided CRISPR-like Immunity in Eukaryotes." Trends Immunol. **40**(11): 998-1010.

Organization, W. H. (1964). "Genetics of Vectors and Insecticide Resistance: Report of a WHO Scientific Group [meeting held in Geneva from 5 to 9 August 1963]."

Ouédraogo, W. M., K. H. Toé, A. Sombié, M. Viana, C. Bougouma, A. Sanon, D. Weetman, P. J. McCall, H. Kanuka and A. Badolo (2022). "Impact of physicochemical parameters of Aedes aegypti breeding habitats on mosquito productivity and the size of emerged adult mosquitoes in Ouagadougou City, Burkina Faso." Parasit. Vectors **15**(1): 478.

Overcash, J. and Z. N. Adelman (2016). Chapter One - Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. Advances in Insect Physiology. A. S. Raikhel, Academic Press. **51:** 1-35.

Palatini, U., P. Miesen, R. Carballar-Lejarazu, L. Ometto, E. Rizzo, Z. Tu, R. P. van Rij and M. Bonizzoni (2017). "Comparative genomics shows that viral integrations are abundant and express piRNAs in the arboviral vectors Aedes aegypti and Aedes albopictus." BMC Genomics **18**(1): 512.

Pane, A., P. Jiang, D. Y. Zhao, M. Singh and T. Schüpbach (2011). "The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline." EMBO J. **30**(22): 4601-4615.

Paradkar, P. N., J.-B. Duchemin, R. Voysey and P. J. Walker (2014). "Dicer-2-dependent activation of Culex Vago occurs via the TRAF-Rel2 signaling pathway." PLoS Negl. Trop. Dis. **8**(4): e2823.

Paradkar, P. N., L. Trinidad, R. Voysey, J.-B. Duchemin and P. J. Walker (2012). "Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway." Proc. Natl. Acad. Sci. U. S. A. **109**(46): 18915-18920.

Park, S. H., M. Cao and G. Bao (2023). "Detection and quantification of unintended large on-target gene modifications due to CRISPR/Cas9 editing." Current Opinion in Biomedical Engineering **28**: 100478.

Parker, J. S., S. M. Roe and D. Barford (2005). "Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex." Nature **434**(7033): 663-666.

Petit, M., V. Mongelli, L. Frangeul, H. Blanc, F. Jiggins and M.-C. Saleh (2016). "piRNA pathway is not required for antiviral defense in Drosophila melanogaster." Proc. Natl. Acad. Sci. U. S. A. **113**(29): E4218-4227.

Phuc, H. K., M. H. Andreasen, R. S. Burton, C. Vass, M. J. Epton, G. Pape, G. Fu, K. C. Condon, S. Scaife, C. A. Donnelly, P. G. Coleman, H. White-Cooper and L. Alphey (2007). "Late-acting dominant lethal genetic systems and mosquito control." BMC Biol. **5**: 11.

Pless, E., A. Gloria-Soria, B. R. Evans, V. Kramer, B. G. Bolling, W. J. Tabachnick and J. R. Powell (2017). "Multiple introductions of the dengue vector, Aedes aegypti, into California." PLoS Negl. Trop. Dis. **11**(8): e0005718.

Poirier, E. Z., B. Goic, L. Tomé-Poderti, L. Frangeul, J. Boussier, V. Gausson, H. Blanc, T. Vallet, H. Loyd, L. I. Levi, S. Lanciano, C. Baron, S. H. Merkling, L. Lambrechts, M. Mirouze, S. Carpenter, M. Vignuzzi and M.-C. Saleh (2018). "Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects." Cell Host Microbe **23**(3): 353-365.e358.

Powell, J. R. and W. J. Tabachnick (2013). "History of domestication and spread of Aedes aegypti--a review." Mem. Inst. Oswaldo Cruz **108 Suppl 1**: 11-17.

Price, T. A. R., N. Windbichler, R. L. Unckless, A. Sutter, J.-N. Runge, P. A. Ross, A. Pomiankowski, N. L. Nuckolls, C. Montchamp-Moreau, N. Mideo, O. Y. Martin, A. Manser, M. Legros, A. M. Larracuente, L. Holman, J. Godwin, N. Gemmell, C. Courret, A. Buchman, L. G. Barrett and A. K. Lindholm (2020). "Resistance to natural and synthetic gene drive systems." J. Evol. Biol. **33**(10): 1345-1360.

Prudêncio, M. (2020). "In Fairness to Mosquitoes." Trends Parasitol. **36**(11): 876-877.

Qu, J., V. Betting, R. van Iterson, F. M. Kwaschik and R. P. van Rij (2023). "Chromatin profiling identifies transcriptional readthrough as a conserved mechanism for piRNA biogenesis in mosquitoes." Cell Rep. **42**(3): 112257.

Riabinina, O., M. Quinn and J. P. Whitehead (2022). "Genetic Toolbox Approaches in Mosquitoes." Cold Spring Harb. Protoc. **2022**(11): Pdb.top107691.

Rivas, F. V., N. H. Tolia, J.-J. Song, J. P. Aragon, J. Liu, G. J. Hannon and L. Joshua-Tor (2005). "Purified Argonaute2 and an siRNA form recombinant human RISC." Nat. Struct. Mol. Biol. **12**(4): 340-349.

Robine, N., N. C. Lau, S. Balla, Z. Jin, K. Okamura, S. Kuramochi-Miyagawa, M. D. Blower and E. C. Lai (2009). "A Broadly Conserved Pathway Generates 3'UTR-Directed Primary piRNAs." Curr. Biol. **19**(24): 2066-2076.

Robinson, A. S. (2005). "Genetic basis of the sterile insect technique." Sterile insect technique: principles and practice in area.

Rose, N. H., A. Badolo, M. Sylla, J. Akorli, S. Otoo, A. Gloria-Soria, J. R. Powell, B. J. White, J. E. Crawford and C. S. McBride (2023). "Dating the origin and spread of specialization on human hosts in Aedes aegypti mosquitoes." Elife **12**.

Rose, N. H., M. Sylla, A. Badolo, J. Lutomiah, D. Ayala, O. B. Aribodor, N. Ibe, J. Akorli, S. Otoo, J.-P. Mutebi, A. L. Kriete, E. G. Ewing, R. Sang, A. Gloria-Soria, J. R. Powell, R. E. Baker, B. J. White, J. E. Crawford and C. S. McBride (2020). "Climate and Urbanization Drive Mosquito Preference for Humans." Curr. Biol. **30**(18): 3570-3579.e3576.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault and M. Solignac (1997). "Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods." Proceedings of the Royal Society of London. Series B: Biological Sciences **250**(1328): 91-98.

Ryan, P. A., A. P. Turley, G. Wilson, T. P. Hurst, K. Retzki, J. Brown-Kenyon, L. Hodgson, N. Kenny, H. Cook, B. L. Montgomery, C. J. Paton, S. A. Ritchie, A. A. Hoffmann, N. P. Jewell, S. K. Tanamas, K. L. Anders, C. P. Simmons and S. L. O'Neill (2019). "Establishment of wMel Wolbachia in Aedes aegypti mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia." Gates Open Res **3**: 1547.

Ryan, S. J., C. J. Carlson, E. A. Mordecai and L. R. Johnson (2019). "Global expansion and redistribution of Aedes-borne virus transmission risk with climate change." PLoS Negl. Trop. Dis. **13**(3): e0007213.

Saito, K., S. Inagaki, T. Mituyama, Y. Kawamura, Y. Ono, E. Sakota, H. Kotani, K. Asai, H. Siomi and M. C. Siomi (2009). "A regulatory circuit for piwi by the large Maf gene traffic jam in Drosophila." Nature **461**(7268): 1296-1299.

Saito, K., H. Ishizu, M. Komai, H. Kotani, Y. Kawamura, K. M. Nishida, H. Siomi and M. C. Siomi (2010). "Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in Drosophila." Genes Dev. **24**(22): 2493-2498.

Saito, K., K. M. Nishida, T. Mori, Y. Kawamura, K. Miyoshi, T. Nagami, H. Siomi and M. C. Siomi (2006). "Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome." Genes Dev. **20**(16): 2214-2222.

Saito, K., Y. Sakaguchi, T. Suzuki, T. Suzuki, H. Siomi and M. C. Siomi (2007). "Pimet, the Drosophila homolog of HEN1, mediates 2'-O-methylation of Piwi- interacting RNAs at their 3' ends." Genes Dev. **21**(13): 1603-1608.

Saiyasombat, R., B. G. Bolling, A. C. Brault, L. C. Bartholomay and B. J. Blitvich (2011). "Evidence of efficient transovarial transmission of Culex flavivirus by Culex pipiens (Diptera: Culicidae)." J. Med. Entomol. **48**(5): 1031-1038.

Salazar, M. I., J. H. Richardson, I. Sánchez-Vargas, K. E. Olson and B. J. Beaty (2007). "Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes." BMC Microbiol. **7**: 9.

Saldaña, M. A., K. Etebari, C. E. Hart, S. G. Widen, T. G. Wood, S. Thangamani, S. Asgari and G. L. Hughes (2017). "Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes." PLoS Negl. Trop. Dis. **11**(7): e0005760.

Samuel, G. H., Z. N. Adelman and K. M. Myles (2018). "Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes." Trends Microbiol. **26**(5): 447-461.

Samuel, G. H., T. Pohlenz, Y. Dong, N. Coskun, Z. N. Adelman, G. Dimopoulos and K. M. Myles (2023). "RNA interference is essential to modulating the pathogenesis of mosquito-borne viruses in the yellow fever mosquito Aedes aegypti." Proc. Natl. Acad. Sci. U. S. A. **120**(11): e2213701120.

Sánchez-Vargas, I., J. C. Scott, B. K. Poole-Smith, A. W. E. Franz, V. Barbosa-Solomieu, J. Wilusz, K. E. Olson and C. D. Blair (2009). "Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway." PLoS Pathog. **5**(2): e1000299.

Schairer, C. E., C. Triplett, O. S. Akbari and C. S. Bloss (2022). "California Residents' Perceptions of Gene Drive Systems to Control Mosquito-Borne Disease." Front Bioeng Biotechnol **10**: 848707.

Schmid-Burgk, J. L., K. Höning, T. S. Ebert and V. Hornung (2016). "CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism." Nat. Commun. **7**: 12338.

Schmidt, T. L., N. H. Barton, G. Rašić, A. P. Turley, B. L. Montgomery, I. Iturbe-Ormaetxe, P. E. Cook, P. A. Ryan, S. A. Ritchie, A. A. Hoffmann, S. L. O'Neill and M. Turelli (2017). "Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti." PLoS Biol. **15**(5): e2001894.

Schnettler, E., C. L. Donald, S. Human, M. Watson, R. W. C. Siu, M. McFarlane, J. K. Fazakerley, A. Kohl and R. Fragkoudis (2013). "Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells." J. Gen. Virol. **94**(Pt 7): 1680-1689.

Schwarz, D. S., Y. Tomari and P. D. Zamore (2004). "The RNA-induced silencing complex is a Mg2+-dependent endonuclease." Curr. Biol. **14**(9): 787-791.

Scott, J. C., D. E. Brackney, C. L. Campbell, V. Bondu-Hawkins, B. Hjelle, G. D. Ebel, K. E. Olson and C. D. Blair (2010). "Comparison of dengue virus type 2-specific small RNAs from RNA interference-competent and -incompetent mosquito cells." PLoS Negl. Trop. Dis. **4**(10): e848.

Scott, T. W., G. G. Clark, L. H. Lorenz, P. H. Amerasinghe, P. Reiter and J. D. Edman (1993). "Detection of multiple blood feeding in Aedes aegypti (Diptera: Culicidae) during a single gonotrophic cycle using a histologic technique." J. Med. Entomol. **30**(1): 94-99.

Scully, R., A. Panday, R. Elango and N. A. Willis (2019). "DNA double-strand break repair-pathway choice in somatic mammalian cells." Nat. Rev. Mol. Cell Biol. **20**(11): 698-714.

Servick, K. (2019). "GM mosquito study draws fire." Science **365**(6459): 1234.

Shanahan, T. M., N. P. McKay, K. A. Hughen, J. T. Overpeck, B. Otto-Bliesner, C. W. Heil, J. King, C. A. Scholz and J. Peck (2015). "The time-transgressive termination of the African Humid Period." Nat. Geosci. **8**(2): 140-144.

Shaw, W. R. and F. Catteruccia (2019). "Vector biology meets disease control: using basic research to fight vector-borne diseases." Nat Microbiol **4**(1): 20-34.

Shi, Y., N. Kopparapu, L. Ohler and D. J. Dickinson (2023). "Efficient and rapid fluorescent protein knock-in with universal donors in mouse embryonic stem cells." Development **150**(10).

Shirai, Y., M. Takahashi, M. Ote, H. Kanuka and T. Daimon (2023). "DIPA-CRISPR gene editing in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae)." Appl. Entomol. Zool. **58**(3): 273-278.

Shousha, A. T. (1948). "Species-eradication: The Eradication of Anopheles gambioe from Upper Egypt, 1942-1945." Bull. World Health Organ. **1**(2): 309-352.

Sienski, G., D. Dönertas and J. Brennecke (2012). "Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression." Cell **151**(5): 964-980.

Sim, S., J. L. Ramirez and G. Dimopoulos (2012). "Dengue virus infection of the Aedes aegypti salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior." PLoS Pathog. **8**(3): e1002631.

Smidler, A. L., O. Terenzi, J. Soichot, E. A. Levashina and E. Marois (2013). "Targeted mutagenesis in the malaria mosquito using TALE nucleases." PLoS One **8**(8): e74511.

Smith, R. H. and R. C. von Borstel (1972). "Genetic control of insect populations." Science **178**(4066): 1164-1174.

Song, J.-J., J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon and L. Joshua-Tor (2003). "The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes." Nat. Struct. Biol. **10**(12): 1026-1032.

Song, J.-J., S. K. Smith, G. J. Hannon and L. Joshua-Tor (2004). "Crystal structure of Argonaute and its implications for RISC slicer activity." Science **305**(5689): 1434-1437.

Souza-Neto, J. A., S. Sim and G. Dimopoulos (2009). "An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense." Proc. Natl. Acad. Sci. U. S. A. **106**(42): 17841-17846.

Spadar, A., J. E. Phelan, E. D. Benavente, M. Campos, L. F. Gomez, F. Mohareb, T. G. Clark and S. Campino (2021). "Flavivirus integrations in Aedes aegypti are limited and highly conserved across samples from different geographic regions unlike integrations in Aedes albopictus." Parasit. Vectors **14**(1): 332.

Sudharshan, S., R. Naidu, M. Mallavarapu and N. Bolan (2012). "DDT remediation in contaminated soils: a review of recent studies." Biodegradation **23**(6): 851-863.

Sun, R., M. Li, C. J. McMeniman and O. S. Akbari (2022). "CRISPR-Mediated Genome Engineering in Aedes aegypti." Methods Mol. Biol. **2509**: 23-51.

Sun, Y. H., L. H. Xie, X. Zhuo, Q. Chen, D. Ghoneim, B. Zhang, J. Jagne, C. Yang and X. Z. Li (2017). "Domestic chickens activate a piRNA defense against avian leukosis virus." Elife **6**.

Suzuki, Y., A. Baidaliuk, P. Miesen, L. Frangeul, A. B. Crist, S. H. Merkling, A. Fontaine, S. Lequime, I. Moltini-Conclois, H. Blanc, R. P. van Rij, L. Lambrechts and M.-C. Saleh (2020). "Non-retroviral endogenous viral element limits cognate virus replication in Aedes aegypti ovaries." Curr. Biol.

Suzuki, Y., L. Frangeul, L. B. Dickson, H. Blanc, Y. Verdier, J. Vinh, L. Lambrechts and M.-C. Saleh (2017). "Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes." J. Virol. **91**(15).

Swarts, D. C., K. Makarova, Y. Wang, K. Nakanishi, R. F. Ketting, E. V. Koonin, D. J. Patel and J. van der Oost (2014). "The evolutionary journey of Argonaute proteins." Nat. Struct. Mol. Biol. **21**(9): 743-753.

Tabachnick, W. J. (1991). "Evolutionary Genetics and Arthropod-borne Disease: The Yellow Fever Mosquito." Am. Entomol. **37**(1): 14-26.

Takahashi, T., S. M. Heaton and N. F. Parrish (2021). "Mammalian antiviral systems directed by small RNA." PLoS Pathog. **17**(12): e1010091.

Takken, W., W. B. Snellen, J. P. Verhave, B. G. J. Knols and S. Atmosoedjono (1990). "Environmental measures for malaria control in Indonesia -an historical review on species sanitation." WAGENINGEN AGRICULTURAL UNIVERSITY PAPERS.

Tassetto, M., M. Kunitomi, Z. J. Whitfield, P. T. Dolan, I. Sánchez-Vargas, M. Garcia-Knight, I. Ribiero, T. Chen, K. E. Olson and R. Andino (2019). "Control of RNA viruses in mosquito cells through the acquisition of vDNA and endogenous viral elements." Elife **8**.

Terenius, O., O. Marinotti, D. Sieglaff and A. A. James (2008). "Molecular genetic manipulation of vector mosquitoes." Cell Host Microbe **4**(5): 417-423.

Thizy, D., L. Pare Toe, C. Mbogo, D. Matoke-Muhia, V. P. Alibu, S. K. Barnhill-Dilling, T. Chantler, G. Chongwe, J. Delborne, L. Kapiriri, E. Nassonko Kavuma, S. Koloi-Keaikitse, A. Kormos, K. Littler, D. Lwetoijera, R. Vargas de Moraes, N. Mumba, L. Mutengu, S. Mwichuli, S. E. Nabukenya, J. Nakigudde, P. Ndebele, C. Ngara, E. Ochomo, S. Odiwuor Ondiek, S. Rivera, A. J. Roberts, B. Robinson, R. Sambakunsi, A. Saxena, N. Sykes, B. B. Tarimo, N. Tiffin and K. H. Tountas (2021). "Proceedings of an expert workshop on community agreement for gene drive research in Africa - Co-organised by KEMRI, PAMCA and Target Malaria." Gates Open Res **5**: 19.

Tungu, P., B. Kabula, T. Nkya, P. Machafuko, E. Sambu, B. Batengana, W. Sudi, Y. A. Derua, V. Mwingira, D. Masue, R. Malima, C. Kitojo, N. Serbantez, E. J. Reaves, C. Mwalimu, S. L. Nhiga, M. Ally, H. R. Mkali, J. J. Joseph, A. Chan, J. Ngondi, S. Lalji, S. Nyinondi, E. Eckert, R. Reithinger, S.

Magesa and W. N. Kisinza (2023). "Trends of insecticide resistance monitoring in mainland Tanzania, 2004-2020." Malar. J. **22**(1): 100.

Unckless, R. L., A. G. Clark and P. W. Messer (2017). "Evolution of Resistance Against CRISPR/Cas9 Gene Drive." Genetics **205**(2): 827-841.

UniProt, C. (2023). "UniProt: the Universal Protein Knowledgebase in 2023." Nucleic Acids Res. **51**(D1): D523-D531.

Vagin, V. V., A. Sigova, C. Li, H. Seitz, V. Gvozdev and P. D. Zamore (2006). "A distinct small RNA pathway silences selfish genetic elements in the germline." Science **313**(5785): 320-324.

van den Berg, H., H. S. da Silva Bezerra, S. Al-Eryani, E. Chanda, B. N. Nagpal, T. B. Knox, R. Velayudhan and R. S. Yadav (2021). "Recent trends in global insecticide use for disease vector control and potential implications for resistance management." Sci. Rep. **11**(1): 23867.

van Rij, R. P., M.-C. Saleh, B. Berry, C. Foo, A. Houk, C. Antoniewski and R. Andino (2006). "The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster." Genes Dev. **20**(21): 2985-2995.

Varjak, M., C. L. Donald, T. J. Mottram, V. B. Sreenu, A. Merits, K. Maringer, E. Schnettler and A. Kohl (2017). "Characterization of the Zika virus induced small RNA response in Aedes aegypti cells." PLoS Negl. Trop. Dis. **11**(10): e0006010.

Varjak, M., K. Maringer, M. Watson, V. B. Sreenu, A. C. Fredericks, E. Pondeville, C. L. Donald, J. Sterk, J. Kean, M. Vazeille, A.-B. Failloux, A. Kohl and E. Schnettler (2017). "Aedes aegypti Piwi4 Is a Noncanonical PIWI Protein Involved in Antiviral Responses." mSphere **2**(3).

Venkataraman, K., N. Shai, P. Lakhiani, S. Zylka, J. Zhao, M. Herre, J. Zeng, L. A. Neal, H. Molina, L. Zhao and L. B. Vosshall (2023). "Two novel, tightly linked, and rapidly evolving genes underlie Aedes aegypti mosquito reproductive resilience during drought." Elife **12**.

Vodovar, N., A. W. Bronkhorst, K. W. R. van Cleef, P. Miesen, H. Blanc, R. P. van Rij and M.-C. Saleh (2012). "Arbovirus-derived piRNAs exhibit a ping-pong signature in mosquito cells." PLoS One **7**(1): e30861.

Wang, W., B. W. Han, C. Tipping, D. T. Ge, Z. Zhang, Z. Weng and P. D. Zamore (2015). "Slicing and Binding by Ago3 or Aub Trigger Piwi-Bound piRNA Production by Distinct Mechanisms." Mol. Cell **59**(5): 819-830.

Wang, Y., B. Jin, P. Liu, J. Li, X. Chen and J. Gu (2018). "piRNA Profiling of Dengue Virus Type 2-Infected Asian Tiger Mosquito and Midgut Tissues." Viruses **10**(4).

Wang, Y., S. Juranek, H. Li, G. Sheng, T. Tuschl and D. J. Patel (2008). "Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex." Nature **456**(7224): 921-926.

Weaver, S. C. and W. K. Reisen (2010). "Present and future arboviral threats." Antiviral Res. **85**(2): 328-345.

Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed and J. J. Welch (2015). "The incidence of bacterial endosymbionts in terrestrial arthropods." Proc. Biol. Sci. **282**(1807): 20150249.

Werren, J. H., L. Baldo and M. E. Clark (2008). "Wolbachia: master manipulators of invertebrate biology." Nat. Rev. Microbiol. **6**(10): 741-751.

Whitfield, Z. J., P. T. Dolan, M. Kunitomi, M. Tassetto, M. G. Seetin, S. Oh, C. Heiner, E. Paxinos and R. Andino (2017). "The Diversity, Structure, and Function of Heritable Adaptive Immunity Sequences in the Aedes aegypti Genome." Curr. Biol. **27**(22): 3511-3519.e3517.

Who (2023). "Geographical expansion of cases of dengue and chikungunya beyond the historical areas of transmission in the Region of the Americas."

Williams, A. E., G. Shrivastava, A. G. Gittis, S. Ganesan, I. Martin-Martin, P. C. Valenzuela Leon, K. E. Olson and E. Calvo (2021). "Aedes aegypti Piwi4 Structural Features Are Necessary for RNA Binding and Nuclear Localization." Int. J. Mol. Sci. **22**(23).

Wilson, A. L., O. Courtenay, L. A. Kelly-Hope, T. W. Scott, W. Takken, S. J. Torr and S. W. Lindsay (2020). "The importance of vector control for the control and elimination of vector-borne diseases." PLoS Negl. Trop. Dis. **14**(1): e0007831.

Wu, H., R. Pang, T. Cheng, L. Xue, H. Zeng, T. Lei, M. Chen, S. Wu, Y. Ding, J. Zhang, M. Shi and Q. Wu (2020). "Abundant and Diverse RNA Viruses in Insects Revealed by RNA-Seq Analysis: Ecological and Evolutionary Implications." mSystems **5**(4).

Wu, P.-H., Y. Fu, K. Cecchini, D. M. Özata, A. Arif, T. Yu, C. Colpan, I. Gainetdinov, Z. Weng and P. D. Zamore (2020). "The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility." Nat. Genet. **52**(7): 728-739.

Xi, Z., J. L. Ramirez and G. Dimopoulos (2008). "The Aedes aegypti toll pathway controls dengue virus infection." PLoS Pathog. **4**(7): e1000098.

Xiao, X., Y. Liu, X. Zhang, J. Wang, Z. Li, X. Pang, P. Wang and G. Cheng (2014). "Complement-related proteins control the flavivirus infection of Aedes aegypti by inducing antimicrobial peptides." PLoS Pathog. **10**(4): e1004027.

Xiol, J., P. Spinelli, M. A. Laussmann, D. Homolka, Z. Yang, E. Cora, Y. Couté, S. Conn, J. Kadlec, R. Sachidanandam, M. Kaksonen, S. Cusack, A. Ephrussi and R. S. Pillai (2014). "RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts." Cell **157**(7): 1698-1711.

Yadav, A. K., C. Butler, A. Yamamoto, A. A. Patil, A. L. Lloyd and M. J. Scott (2023).

"CRISPR/Cas9-based split homing gene drive targeting doublesex for population suppression of the global fruit pest Drosophila suzukii." Proc. Natl. Acad. Sci. U. S. A. **120**(25): e2301525120.

Yamaguchi, S., A. Oe, K. M. Nishida, K. Yamashita, A. Kajiya, S. Hirano, N. Matsumoto, N. Dohmae, R. Ishitani, K. Saito, H. Siomi, H. Nishimasu, M. C. Siomi and O. Nureki (2020). "Crystal structure of Drosophila Piwi." Nat. Commun. **11**(1): 858.

Yang, W. and T. A. Steitz (1995). "Recombining the structures of HIV integrase, RuvC and RNase H." Structure **3**(2): 131-134.

Yao, F. A., A.-A. Millogo, P. S. Epopa, A. North, F. Noulin, K. Dao, M. Drabo, C. Guissou, S. Kekele, M. Namountougou, R. K. Ouedraogo, L. Pare, N. Barry, R. Sanou, H. Wandaogo, R. K. Dabire, A. McKemey, F. Tripet and A. Diabaté (2022). "Mark-release-recapture experiment in Burkina Faso demonstrates reduced fitness and dispersal of genetically-modified sterile malaria mosquitoes." Nat. Commun. **13**(1): 796.

Yen, J. H. and A. R. Barr (1973). "The etiological agent of cytoplasmic incompatibility in Culex pipiens." J. Invertebr. Pathol. **22**(2): 242-250.

Yuan, Y.-R., Y. Pei, J.-B. Ma, V. Kuryavyi, M. Zhadina, G. Meister, H.-Y. Chen, Z. Dauter, T. Tuschl and D. J. Patel (2005). "Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage." Mol. Cell **19**(3): 405-419.

Zhang, F., J. Wang, J. Xu, Z. Zhang, B. S. Koppetsch, N. Schultz, T. Vreven, C. Meignin, I. Davis, P. D. Zamore, Z. Weng and W. E. Theurkauf (2012). "UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery." Cell **151**(4): 871-884.

Zheng, K. and P. J. Wang (2012). "Blockade of pachytene piRNA biogenesis reveals a novel requirement for maintaining post-meiotic germline genome integrity." PLoS Genet. **8**(11): e1003038.

Zheng, X., D. Zhang, Y. Li, C. Yang, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, J. Bouyer, M. Tang, B. Zheng, J. Yu, J. Liu, J. Zhuang, Z. Hu, M. Zhang, J.-T. Gong, X.-Y. Hong, Z. Zhang, L. Lin, Q. Liu, Z. Hu, Z. Wu, L. A. Baton, A. A. Hoffmann and Z. Xi (2019). "Incompatible and sterile insect techniques combined eliminate mosquitoes." Nature **572**(7767): 56-61.

Zhu, G.-H., N. M. Albishi, X. Chen, R. L. Brown and S. R. Palli (2021). "Expanding the Toolkit for Genome Editing in a Disease Vector, Aedes aegypti: Transgenic Lines Expressing Cas9 and Single Guide RNA Induce Efficient Mutagenesis." CRISPR J **4**(6): 846-853.

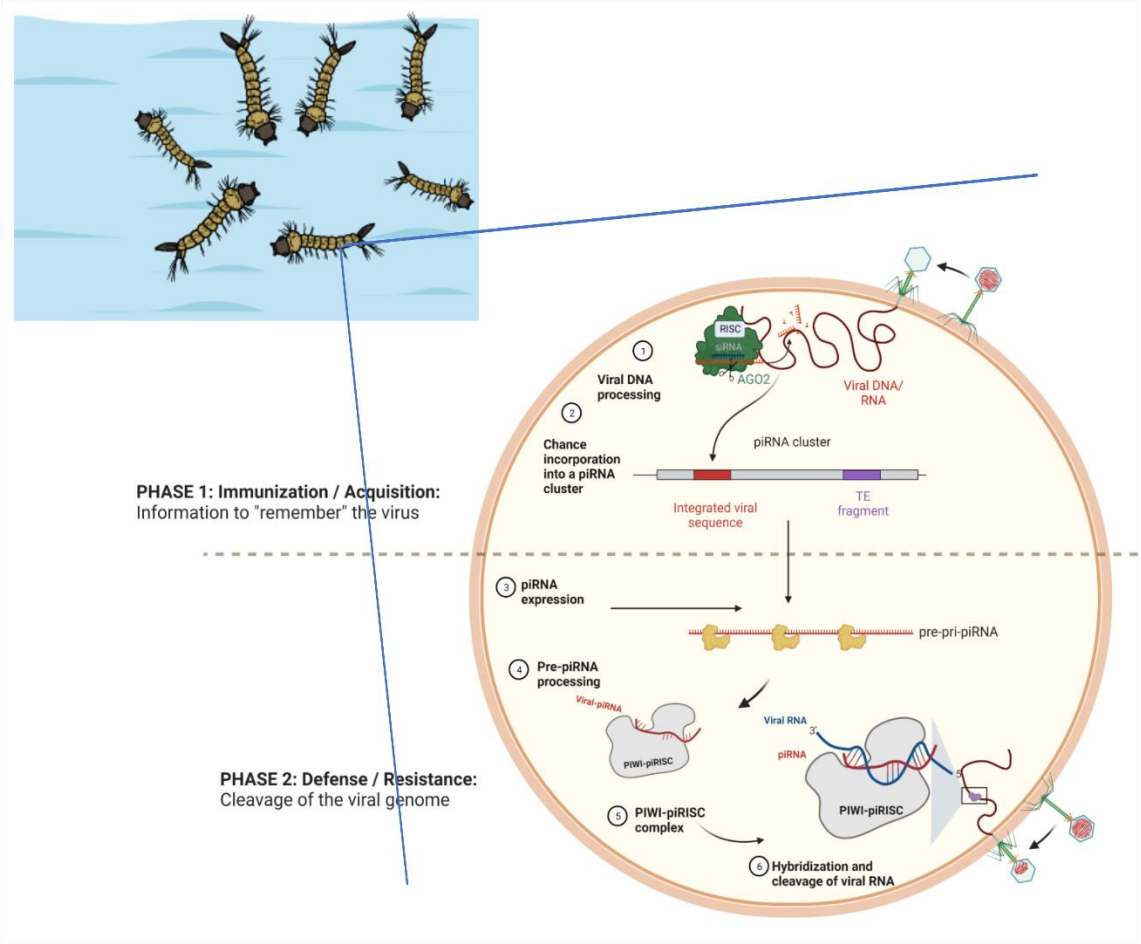


**Figure 5.1:** The adaptive immune system in Ae. aegypti. The piRNA pathway evolved as immunity against entomopathogenic viruses that infect larvae. As the larvae feed on decaying organic matter in water, viruses may be ingested. The virus enters the cells of the midgut epithelium, activating both the siRNA and piRNA pathways. Chance insertion of viral-derived sequences into a piRNA cluster leads to an EVE-derived viral-piRNA response during the next encounter with the cognate virus. EVE-derived piRNAs work in concert with the siRNA pathway to limit the replication of the virus.