UNIVERSITY OF CALIFORNIA SAN DIEGO

Improving genetic control strategies for insect vector control

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

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2020

The Dissertation of Stephanie Gamez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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2020

DEDICATION

I dedicate this dissertation to my parents who have sacrificed and risked their lives crossing the Mexico-U.S. border to give their children the opportunity for a better life. This is for my mother, **Maria** who gave up her nursing passion and education so that I can pursue success and find my passion in science. This is for my father, **Armando** who worked so much for too long to make sure our family did not struggle financially. Your sacrifice was not in vain, *apa*. This is for my brother **Armando Jr.**, who always dedicated time to sooth my emotional pain and for his dedication to ensure my sister and I continued our education. This is for my sister **Liz**, whose intelligence and love gave me the strength to pursue something greater. You and I stand on the shoulders of Giants. This is for my brother **Alex**, who lifted our spirits when life was getting tough. You are the spark and joy of our family. This is for my brother **Raul** who, despite distance and separation, welcomed my family into his life with open arms. This is for my partner, **Matt**, whose never ending love and support helped me grow into a confident and better person.

I thank my mentor, Omar Akbari, for pushing me to be the best I can be. Thank you for giving me many opportunities to better myself professionally and giving me the confidence to pursue greater things. I also thank my committee members for their patience, knowledge, and kindness throughout my PhD career.

Finally, this is for my professors, mentors, teachers, family and friends throughout my life who believed in my ability to be successful when I couldn't see it. My successes are also yours.

EPIGRAPH

“Inspiration gives no warnings.”

Gabriel Garcia Marquez

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LIST OF ABBREVIATIONS

ZIKV	Zika virus
IIT	Incompatible insect technique
SIT	Sterile Insect Technique
CI	Cytoplasmic incompatibility
CHIKV	Chikungunya virus
YFV	Yellow fever virus
DENV	Dengue virus
MAYV	Mayaro virus
WNV	West Nile virus
GD	Gene Drive
NHEJ	Nonhomologous end-joining
HGD	Homing-based gene drives
gRNA	guide RNA
RIDL	Release of Insects with a Dominant Lethal gene
ClvR	Cleave and rescue
RNAi	RNA interference
WT	Wildtype
iPCR	Inverse polymerase chain reaction
qPCR	Quantitative PCR
esiRNA	endogenous small RNAs
TPM	Transcripts per million
$TCID_{50}$	Median tissue culture infectious dose

PEV	Position effect variegation
dpi	Days post infection
CP	Carboxypeptidase promoter
SDD	Severe dengue disease
MAb	Monoclonal antibody
scFv	Single-chain variable fragment
CPA	Carboxypeptidase promoter (*Aedes aegypti*)
*w*Mel	Wolbachia Mel strain
GE	Genome equivalents
PBM	Post blood meal
NBF	Non-blood fed
PCA	Principal component analysis
GO	Gene ontology
hr	hours
FPKM	Fragments per kilobase of transcript per million mapped reads

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ACKNOWLEDGEMENTS

I would like to acknowledge the following people:

Dr. Omar S. Akbari for his support as the chair of my committee and my mentor. His enthusiasm, guidance, patience, and support were crucial for my professional development.

Dr. Anna Buchman for her support, guidance, and mentorship during the first couple years of my graduate career. Thank you for helping me be the person I am today. I will never forget.

Dr. Robyn Raban for her expertise in all things virus into the lab and for helping me on personal matters. You've been extraordinarily supportive throughout my career and I appreciate that.

Dr. Ming Li for his kindness, support, and for injecting all the mosquito transgenic lines included in this dissertation. You have a unique skill that cannot be topped!

Dr. Andie Smidler for her profound kindness, silliness, and intelligence. Thank you for taking the time to have career conversions with me and for helping me prepare for my presentation. You are delightful to be around with.

Judy Ishikawa for her enormous support in the mosquito insectary and for being a wonderful and kind friend.

The graduate students including Michelle Bui, Dan Brogan, and Junru Liu for their strong friendship and support in work and personal matters. You guys made graduate school worth it.

Dr. Igor Antoshechkin for his expertise in all things bioinformatics and RNA-Sequencing. Thank you for being very patient with my bioinformatics questions and requests.

Dr. Prasad N. Paradkar, Dr. Jean-Bernard Duchemin, and Melissa J. Klein for their help in setting up mosquito infection experiments and providing valuable insight into our results.

Dr. Chun-Hong Chen, Hsing-Han Li, and Hsin-Wei Wang for their support from Taiwan.

I would like to thank Dr. James Crowe for allowing us to utilize his 1C19 antibody for the generation of anti-DENV mosquitoes.

Both Dr. Stelia C. Mendez-Sanchez and Luis C. Vesga for their help on the proteomics aspect of my projects.

Both Dr. Jonny Duque and Dr. Stelia C. Mendez-Sanchez for their support and mentorship throughout their stay in the Akbari lab. You both gave me confidence in my scientific abilities.

Dr. John Marshall, Luke Alphey, and Jason Rasgon for their incredible support and advice throughout the writing process of our review. Thank you for taking the time to help us improve our work.

My committee members, Dr. Ethan Bier, Dr. Elizabeth Winzeler, Dr. Justin Meyer, and Dr. Mark Estelle for their encouragement, patience, and helpful insights. You are all awesome mentors.

Finally, I would like to thank Dr. Guanhong Wang for his kindness and support throughout the writing process of our review and my current and former lab mates including Dr. Duverney Chaverra Rodriguez, Lenissa Alcantara, Tyler Wise, Dr. Cody Gilleland, Dr. Elena Dalla Benetta, Dr. Ting Yang, Isaiah Shriner, Dr. Nikolay Kandul, Hoa Nguyen, Spencer Louie, Christian Bowman, Dr. Sitara Roy, Joan Chung, Jennifer Shyong, Rose Oshana, Dennis Ostrovsky, Wenkang Feng, Jiaxin Luo, Nicole Colindres, Dr. Jackson Champer, and Parker Hill.

The material in this dissertation is based on the following papers which are either published, submitted for publication, or preparing for submission.

Chapter 1, in full, is a reprint of the material that was submitted for publication. Guan-Hong Wang[1], Stephanie Gamez[1], Robyn Raban., John M. Marshall., Luke Alphey, Ming Li, Jason L. Rasgon., Omar S. Akbari. Combating mosquito-borne pathogens using genetic control technologies. (2020) Submitted to Nature Biotechnology [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

Chapter 2, in full, is a reprint of the material as it appears in the Proceedings of the National Academy of Sciences of the United States of America (PNAS). Anna Buchman[1], Stephanie Gamez[1], Ming Li, Igor Antoshechkin, Hsing-Han Li, Hsin-Wei Wang, Chun-Hong Chen, Melissa J. Klein, Jean-Bernard Duchemin, Prasad N. Paradkar, and Omar S. Akbari. "Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic miRNAs". PNAS. (2019). [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

Chapter 3, in full, is a reprint of the material as it appears in PLoS Pathogens. Anna Buchman[1], Stephanie Gamez[1], Ming Li, Igor Antoshechkin, Hsing-Han Li, Hsin-Wei Wang, Chun Hong Chen, Melissa J. Klein, Jean-Bernard Duchemin, James E. Crowe, Jr., Prasad N. Paradkar, and Omar S. Akbari. "Broad Dengue Neutralization in Mosquitoes Expressing an Engineered Antibody". PLoS Pathogens. (2020). [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

Chapter 4, in full, is a reprint of the material as it appears in G3 (Genes, Genomes, Genetics). Stephanie Gamez, Igor Antoshechkin, Stelia Mendez-Sanchez, Omar S. Akbari. A comprehensive developmental transcriptome analysis of *Ae. albopictus*, an invasive mosquito. G3. (2020). The dissertation/thesis author was first author and primary investigator of this paper.

Chapter 5, in full, is a copy of the material that is currently being prepared for publication. Stephanie Gamez, Luis C. Vesga, Stelia C. Mendez-Sanchez, Omar S. Akbari. "Development of alternative transactivational binary expression systems in *Drosophila melanogaster*." The dissertation/thesis author was first author and primary investigator of this paper.

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" A comprehensive developmental transcriptome analysis of *Ae. albopictus*, an invasive mosquito" G3. (2020).

"Translating Gene Drive Science to Include Linguistically Diverse Populations in Community and Stakeholder Engagement" Global Public Health. (2020).

"Broad Dengue Neutralization in Mosquitoes Expressing an Engineered Antibody" Plos Pathogens. (2020).

"Development of a Confinable Gene-Drive System in the Human Disease Vector, *Aedes aegypti*" eLife. (2019).

"Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic miRNAs" PNAS. (2019).

"Genomic insights into adaptive divergence and speciation among malaria vectors of the *Anopheles nili* group" Evolutionary Applications. (2017).

"Pollutants and insecticides drive local adaptation in African malaria mosquitoes" Molecular Biology and Evolution. (2017).

"Extensive genetic diversity among populations of the malaria mosquito *Anopheles moucheti* revealed by population genomics" Infection, Genetics, and Evolution. (2016).

"Polymorphism in an inbred line of the african malaria mosquito *Anopheles gambiae*" Genome Biology and Evolution (2014).

FIELDS OF STUDY

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ABSTRACT OF THE DISSERTATION

Improving genetic control strategies for insect vector control

by

Stephanie Gamez

Doctor of Philosophy in Biology

University of California San Diego, 2020

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The burden of mosquito-borne diseases is a problem that needs to be addressed with better tools. The use of genetics for their control is a promising strategy that has advantages to the use of traditional control methods. However, more work needs to be done before we could implement genetic strategies as actual control approaches in the field. For population modification

approaches, effective effectors need to be developed to target different pathogens and strains of a pathogen. In order to be effective, these effectors need to be able to confer at least 100% pathogen resistance with a minimal fitness impact on the transgenic insect. For population suppression approaches, such as RIDL, transgenic insects cannot have their fitness compromised by tetracycline because their effectiveness depends on the competition between wildtype males to mate with females. The first aim of my thesis involved engineering novel and effective effector transgenes to generate pathogen-resistant mosquitoes. Two different effectors (targeting the ZIKV and four DENV serotypes) were generated that conferred up to 100% resistance to the pathogen. The second aim was to generate a comprehensive developmental transcriptome of *Aedes albopictus*, an understudied mosquito capable of causing mosquito-borne epidemics. This transcriptome will not only provide a valuable molecular resource for others, but it will aid in the development of effective genetic control strategies against this mosquito. Finally, the final aim of my thesis involved developing and testing novel conditional transactivatable systems for controlling gene expression in the model organism, *Drosophila melanogaster*. The results of this work show five prokaryotic operon systems (that were never tested in an animal model) are useful as transactivational systems and for genetic circuits.

Chapter 1: Introduction

1.1 The need for novel control tools against mosquitoes

Roughly half of the world's population is at risk of mosquito-borne diseases, with the highest burden for socioeconomically disadvantaged populations (World Health Organization 2010). Urbanization, globalization, climate change, and land-use shifts have contributed to the re-emergence and expansion of mosquito-borne diseases (Organization and Others 2017). For example, outbreaks of chikungunya, yellow fever, and malaria have increased in size and frequency since 2014 (Organization and Others 2017). Dengue incidence has increased >30-fold in the past 50 years (Pang, Mak, and Gubler 2017), with a current annual infection rate exceeding 400 million (Bhatt et al. 2013). The 2015-2016 Zika virus (ZIKV) outbreak in Latin America and the Caribbean resulted in hundreds of thousands of infections, with large-scale socioeconomic disruption (World Health Organization 2016). Supply-chain disruptions due to the coronavirus disease 2019 pandemic are expected to double the number of malaria-related deaths in sub-Saharan Africa in 2020 as compared to 2018 (World Health Organization 2020).

There is a critical need for safe, sustainable approaches to reduce the burden of mosquito-borne pathogens. Common mosquito control strategies with chemical insecticides and environmental management (Schreck 1991) are only moderately effective, in part due to genetic and behavioral vector resistance (Succo et al. 2016). Chemical interventions may also have unintended effects on nontarget insects, including pollinators (Ware 1980).

Recent years have seen an expansion in genetic control technologies, involving the release of modified mosquitoes to achieve population suppression (Figure 1.1A) or population modification (Figure 1.1B). Suppression strategies include sterile insect technique (SIT), incompatible insect technique (IIT), and various transgene-based technologies. In population

modification strategies, pathogen-resistant ('refractory') mosquitoes are designed and released into wild populations, where they spread their heritable modifications to prevent pathogen transmission. Examples include use of the pathogen-blocking *Wolbachia* and various transgenic technologies. In this review, we compare mosquito control interventions aimed at either population suppression or modification, highlighting recent developments in the use of *Wolbachia*-infected mosquitoes and transgenic strategies.

1.2 *Wolbachia*-based approaches for mosquito control

Wolbachia are intracellular reproductive parasites in arthropods and nematodes, found in ~60% of all insects (Hilgenboecker et al. 2008). Transmitted vertically from mother to offspring, *Wolbachia* maximize their transmission by manipulating host reproduction through feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (CI) (Werren, Baldo, and Clark 2008). Through CI, *Wolbachia*-infected females produce viable *Wolbachia*-infected offspring when mated with infected or uninfected *Wolbachia* males. *Wolbachia*-infected males only produce viable offspring when mated with females infected with the same *Wolbachia* strain. Thus, although males are dead ends for *Wolbachia* transmission, they reduce the population fitness of uninfected females, giving *Wolbachia*-infected females a relative reproductive advantage and enabling infection spread. Interestingly, some important vector species, including *Aedes aegypti*, are naturally free of *Wolbachia* (Ross et al. 2020). Therefore, to use *Wolbachia* to control pathogens transmitted by *Wolbachia*-uninfected mosquitoes, a *Wolbachia* strain must be introduced through microinjection to establish a *Wolbachia*-infected colony (Fraser et al. 2017).

1.3 *Wolbachia*-based population suppression

The principle of population suppression strategies is simple: reduce the size of mosquito populations to prevent disease spread. In the *Wolbachia* IIT strategy, *Wolbachia*-infected male mosquitoes are released into a wild population lacking that *Wolbachia* strain. Any pairings with *Wolbachia*-infected males will result in nonviable offspring. Multiple releases over time can suppress mosquito populations and potentially interrupt disease transmission (Figure 1.1A). Ideal strains for *Wolbachia* IIT should have high penetrance of sterility in matings between *Wolbachia*-infected males and wild females, and should ensure similar mating competitiveness between *Wolbachia*-infected and wild males. Several *Wolbachia* strains that satisfy these conditions have been successfully transinfected into *Aedes*, *Anopheles*, and *Culex* mosquitoes (Zheng et al. 2019; Crawford et al. 2020; Fraser et al. 2017; Gomes and Barillas-Mury 2018; T. H. Ant et al. 2020).

Despite advances, several drawbacks of IIT limit its long-term sustainability. Most importantly, accidental release of *Wolbachia*-infected females into a population can compromise the strategy. Over time, *Wolbachia*-infected females can become more abundant, rendering the *Wolbachia* strain obsolete for mosquito control due to compatibility between infected females and infected males. As with other sterile-male methods, IIT requires continued mosquito releases, necessitating substantial efforts to mass rear and release mosquitoes. Ongoing improvements in efficient mass-rearing and sex-sorting methods can mitigate these issues (Crawford et al. 2020; Zheng et al. 2019). For example, to overcome the issue of sex-sorting methods not being 100% efficient, scientists recently modified the protocol of IIT in *Aedes albopictus* (Figure 1.1A) (Zheng et al. 2019) by sterilizing all sorted pupae with low-dose radiation. While this may compromise the fitness of the released males too, this strategy sterilizes any accidentally released *Wolbachia*-infected females and prevents them from transmitting the *Wolbachia* strain to their offspring which

is essential. This environmentally safe and nonpersistent sterilization method has been used for many years to suppress pest insect populations in SIT programs, whereby chromosomal damage or lethal mutations are introduced to produce nonviable offspring (Knipling 1955).

Recently, researchers at Verily Life Sciences described an automated approach for the mass rearing, sex-sorting, and release of *Wolbachia*-infected *Ae. aegypti* (Crawford et al. 2020) for IIT. After mass rearing of mosquito larvae, male and female pupae are separated using an automated mechanical sieve, based on the sexually dimorphic pupal size in this species. Adult males are verified by machine learning, and then separated by an air jet and shutter system. Each mosquito is individually imaged and selected by male-specific anatomical features before distribution into release tubes. Human and machine-learning classifiers score all male-labeled images and discard female-containing tubes. Finally, a computer-controlled automated system manages tube transport and release. This system generates a map-based release plan and uses a structured light mosquito counter to measure the density of released males. Overall, these improvements should greatly increase the efficiency and applicability of strategies for mosquito control.

1.4 *Wolbachia*-based population modification

Population modification approaches include release of mosquitoes that harbor heritable factors that reduce or block pathogen transmission. For example, *Wolbachia* has been demonstrated to reduce transmission of multiple arboviruses (i.e., dengue, West Nile, chikungunya, Zika, and Mayaro) and even the malaria parasite *Plasmodium* (Dutra et al. 2016; Flores et al. 2020; Pereira et al. 2018). Studies have suggested that *Wolbachia* may block pathogens by competing for fatty acids, regulating host microRNAs, or upregulating innate immune response pathways (Geoghegan et al. 2017), or may interact directly with viral RNA to limit pathogen

infection, dissemination, and transmission (Bhattacharya, Newton, and Hardy 2020). The precise mechanism for the anti-pathogen transmission activity of *Wolbachia* remains unclear and likely varies by host, *Wolbachia* strain, and pathogen.

Field trials of *Wolbachia*-based population modification in isolated pilot locations in Australia demonstrated that *Wolbachia* infection rates reached up to 90% at 11 weeks following an initial release of *Wolbachia*-infected mosquitoes (Ryan et al. 2019). High *Wolbachia*-infection rates were also sustained for 6 months after release in dengue virus (DENV)-endemic Yogyakarta, Indonesia, where *Wolbachia*-infected adults or eggs were released over 20 or 24 weeks, respectively (Tantowijoyo et al. 2020). *Wolbachia*-based population modification strategies require fewer releases than IIT strategies, and allow the release of both sexes of *Wolbachia*-infected mosquitoes, enabling more efficient production by eliminating the need for sex-sorting (Figure 1.1B).

Notwithstanding these advantages, some *Wolbachia* strains have been shown to enhance infection of certain pathogens in mosquitoes. For example, the *Wolbachia* strain *w*AlbB enhanced *Plasmodium* infection in *Anopheles gambiae* (Hughes et al. 2012). Other studies found that the *w*Mel strain may enhance infections of insect-specific flaviviruses (Amuzu et al. 2018) and DENV in *Ae. aegypti* (King et al. 2018). These findings underscore the need for caution when evaluating *Wolbachia*-based approaches for mosquito control.

1.5 Genetic mechanism of CI

Although the *Wolbachia* CI phenomenon has been known for decades (Laven 1951), the genetic basis was recently resolved (Chen et al. 2019; LePage et al. 2017; Bonneau et al. 2018; Beckmann, Ronau, and Hochstrasser 2017). In accordance with the toxin-antidote model (Poinsot,

Charlat, and Mercot 2003), *Wolbachia* introduces a toxin into male sperm, and an antidote is present in eggs of *Wolbachia*-infected females. Embryos die when sperm containing toxin enters an egg lacking the antidote. Embryos survive when a toxin-containing sperm enters an antidote-containing egg.

The first identification of CI-related genes came from a proteomics study, which hypothesized that a *Wolbachia* CI toxin protein should be present in sperm (Beckmann and Fallon 2013). Recently, researchers determined that an operon with two CI-inducing deubiquitylating (DUB) genes, *cidA* and *cidB,* expressed in transgenic male flies can recapitulate the CI phenomenon. The CI phenotype was rescued by mating these transgenic males with a *cidA*-expressing female (Beckmann, Ronau, and Hochstrasser 2017). Two additional CI-factor, DUB-encoding genes, *cifA* and *cifB,* were discovered using comparative genomics (LePage et al. 2017). Interestingly, not all CI-inducing *Wolbachia* have DUB operons. A recent report described an alternative CI system that contains a paralogous *cid* operon containing two CI-inducing genes, *cinA* and *cinB,* which encode for a binding protein and a nuclease, respectively (Chen et al. 2019).

CI-inducing genes are usually located within genomes of WO prophage (Beckmann et al. 2019), a phage that can infect and transform *Wolbachia* genomes (Wang et al. 2016). Although this prophage was identified 20 years ago, WO prophage-*Wolbachia* interactions are only now beginning to be understood. Most obligate intracellular bacteria lack phage infections (Wang et al. 2016), whereas *Wolbachia* can have multiple WO prophage infections simultaneously (Kent and Bordenstein 2010). WO prophage contains both CI-inducing genes and male-killing genes (e.g., *wmk* (Perlmutter et al. 2019)), suggesting an evolutionary advantage for phage infection in *Wolbachia*. The correlation between multiple copies of CI-inducing genes and a stronger CI

phenotype opens an exciting research opportunity to explore how and why *Wolbachia* maintains multiple infections (Kent and Bordenstein 2010).

1.6 Transgenic approaches for mosquito control

The biased transmission by *Wolbachia* is mechanistically similar to that of a gene drive (GD), a 'selfish' genetic system that can spread through populations by biasing inheritance in its favor (J. Champer, Buchman, and Akbari 2016; Raban, Marshall, and Akbari 2020). While varying dramatically in their mechanisms, all GDs selfishly enable their spread without necessarily conferring selective advantage on their carriers. This aspect is important for genetic control of mosquitoes, as mosquito-borne pathogens generally have little adverse effect on infected mosquitoes. Thus, refractory genes, which impart pathogenic resistance, are unlikely to confer significant fitness advantage to carriers. Although inundative release may be sufficient for some purposes (Rasgon 2009), refractory genes generally need to be linked to GDs for large-scale use.

Scientists are developing synthetic GDs, which are often mechanistically inspired by natural GDs (e.g., *Medea* (Beeman and Friesen 1999), homing endonucleases (Burt 2003)) but developed from scratch, allowing them to be better understood and tailored for specific pathogens. There are several GD types with differing characteristics. As well as homing-based gene drives (HGDs) (Ming Li et al. 2020; Hoermann et al. 2020; Pham et al. 2019) and sex-linked meiotic drives (Galizi et al. 2014, 2016), which have been demonstrated in mosquitoes, other GD types include *Medea* and various underdominance systems (A. B. Buchman et al. 2018; Akbari et al. 2013; A. Buchman, Shriner, et al. 2020).

Despite the promise of HGDs for solving world health issues, there are safety concerns due to the potential ability of HGDs to invade non-target populations. To address this, scientists have

proposed the use of split homing-based gene drive (split HGD) as an alternative to autonomous HGD (DiCarlo et al. 2015; Akbari et al. 2015; Kandul, Liu, Buchman, et al. 2020; Ming Li et al. 2020; J. Champer et al. 2020) (See Figure 1.2F). In an autonomous drive, a single unit comprising Cas9 and guide RNA (gRNA) is inserted at a target location (López Del Amo et al. 2020). Split drives separate the CRISPR components across two or more genetic loci, with one component unable to drive. Large-scale releases can allow a split HGD to reach long-term high prevalence in the target population. However, limited introductions (e.g., accidental release (Akbari et al. 2015) or migrants reaching non-target populations) cannot reach high prevalence, because the nondriving component is limited and declines from a low initial frequency due to selection. In this way, split HGDs enable spatiotemporal confinement of HGD elements (Ming Li et al. 2020). Split HGDs have reduced spreading ability relative to equivalent autonomous drives, especially at low prevalence; however, this issue can be substantially restored by linking several elements together if desired (Noble et al. 2019).

Recent advances (Matthews et al. 2018; Ming Li et al. 2017) enabled development of a split HGD in an *Aedes* mosquito (Ming Li et al. 2020). Li et al. examined drive dynamics of a GD element comprising a gRNA targeting a phenotypic gene, *white*, together with an unlinked source of germline Cas9. Researchers achieved inheritance rates of up to 94%. This proof-of-principle study paves the way for further development of linked effectors for population modification strategies or transgenes useful for population suppression in *Ae. aegypti*.

1.7 Population suppression

Several studies have demonstrated HGD suppression systems in *Anopheles* mosquitoes (A. Hammond et al. 2016; Marinotti et al. 2013; Windbichler et al. 2011; Kyrou et al. 2018; Simoni et

al. 2020). A CRISPR HGD was designed to target female fertility genes of *An. gambiae*, to decrease both female reproductive output and mosquito population size (A. Hammond et al. 2016). In another, a CRISPR-based HGD targeting *doublesex (dsx)* eliminated laboratory cage populations of *An. gambiae* (Kyrou et al. 2018) (Figure 1.2A). This system was designed to prevent resistant-allele accumulation at the target site, which is an issue for CRISPR-based GDs (KaramiNejadRanjbar et al. 2018; Unckless, Clark, and Messer 2017; J. Champer et al. 2017; A. M. Hammond et al. 2017; Kandul, Liu, Buchman, et al. 2020), by taking advantage of the highly conserved nature of *dsx*. An alternative GD design, incorporating a previously characterized X-chromosome shredding nuclease, I-PpoI, to the CRISPR-based *dsx* GD system (Simoni et al. 2020) (Figure 1.2B). Experiments demonstrated a biased sex ratio towards males and eventual collapse of a small laboratory cage population. These innovative studies demonstrate the versatility of new technologies to overcome previous limitations.

GD is not the only approach for population suppression. Release of Insects carrying a Dominant Lethal gene (RIDL) has been used for many years for control of insect pests, including mosquitoes (Fu et al. 2010; Labbé et al. 2012) (Figure 1.2C and 1.2D; Table 1.1). Although we will not focus on RIDL here, we recommend the following reviews (Black, Alphey, and James 2011; Alphey et al. 2013; Leftwich, Bolton, and Chapman 2016). Despite successes with RIDL, costs associated with mass rearing using tetracycline (Moullan et al. 2015; Coon, Brown, and Strand 2016) and fitness costs associated with the initial strains have compelled researchers to innovate other non-GD technologies and RIDL strains with lower fitness costs.

A promising non-GD approach for mosquito control, precision-guided sterile insect technique (pgSIT), takes advantage of efficient CRISPR-mediated biallelic lethal/sterile mosaicism to produce sterile males and dead/intersex females (Kandul et al. 2019). Demonstrated

in fruit flies, this approach circumvents the fitness costs associated with SIT and RIDL. The pgSIT study characterized three dual-gRNA strains, all targeting the *beta-2-tubulin* gene and one of three sex determination genes: *sex lethal*, *transformer*, or the female isoform of *dsx*. When these dual gRNA strains were crossed to three Cas9 strains, male progeny were sterile, and females were either dead or converted to sterile intersex. Importantly, pgSIT males were able to compete with wild-type males for females. This approach provides an exciting opportunity for application in mosquitoes, because genes like *beta-2-tubulin*, *dsx* are conserved in mosquitoes (Figure 1.2E). In *Ae. aegypti*, for example, characterization of U6 promoters for gRNA expression (Ming Li et al. 2020) and Cas9 strains (Ming Li et al. 2017) will help streamline the engineering and characterization of an efficient pgSIT approach. This technology works in early embryogenesis, allowing release of eggs (rather than fragile adult mosquitoes) into the field.

1.8 Population modification

Using synthetic or naturally occurring effector genes to reduce pathogen transmission is another approach for mosquito-borne disease control. One concern with population suppression strategies (Hayes et al. 2018) is that eradicating mosquito populations may lead to reinvasion from a neighboring population or another species that occupies the same ecological niche. Population modification approaches are a sustainable and cost-effective means of maintaining local elimination of pathogen-susceptible mosquitoes, while providing a barrier to prevent such reinvasion. In 2015, a population modification approach was developed using a HGD linked to an antimalarial effector in *Anopheles stephensi* (Gantz et al. 2015), achieving super-Mendelian inheritance of the effector gene, however both fitness costs and drive resistant alleles immediately appeared limiting utility of this approach. Recently, a recoded HGD rescue system in *Anopheles stephensi* was developed to relieve the fitness costs associated with a nonfunctional target site and

prevent the formation of resistance alleles and this system showed promising performance in multi-generational laboratory population cages (Adolfi et al. 2020) (Figure 1.2G). Another study demonstrated an effector-linked HGD using minimal genetic modifications in malaria mosquitoes (Hoermann et al. 2020) (Figure 1.2H). Proof-of-principle experiments revealed that an effector construct containing homology arms to an endogenous gene and an artificial intron (gRNA and fluorescent marker) within the effector resulted in 99% of individuals inheriting the construct. This study provides an alternative approach towards designing HGDs with minimal components to decrease associated fitness costs and increase drive efficiency of anti-pathogen effectors. Additional nonhoming based designs for modification include toxin-antidote CRISPR GD systems, such as 'cleave and rescue' elements (*ClvR*) (Oberhofer, Ivy, and Hay 2019, [b] 2020, [a] 2020, 2018) and 'TARE' drives (J. Champer et al. 2020), which work by disrupting an endogenous essential gene and rescuing cleaved individuals by providing an external cleavage-resistant essential gene (recoded gene). These nonhoming GD designs are able to spread and overcome resistance-related challenges. Although *ClvR* has only been demonstrated as a proof-of-principle in fruit flies, this GD has the potential to become a promising strategy for future mosquito modification approaches.

To date, effectors have been engineered to over-express endogenous transcription factors from innate immune pathways (i.e., Toll, IMD, and JAK-STAT) (Bian et al. 2005; Jupatanakul et al. 2017; Corby-Harris et al. 2010; Volohonsky et al. 2017; Dong, Simões, and Dimopoulos 2020), or to express synthetic effectors, such as single-chain antibodies (Sumitani et al. 2013), antiviral hammerhead ribozymes (Mishra et al. 2016), and small RNAs that target mosquito-borne viruses via the host RNA interference (RNAi) pathway (Franz et al. 2006; A. Buchman et al. 2019; Yen et al. 2018). Many early iterations of these effectors, however, were limited in their ability to target

multiple pathogens or parts of the pathogen lifecycle. Recently engineered strategies to address these critical issues have included multistage effector transgenes against different life stages of *Plasmodium falciparum* in *A. stephensi* (Dong, Simões, and Dimopoulos 2020) (Figure 1.2I), a dual-antiviral effector targeting two distinct viral families (Yen et al. 2018) (Figure 1.2J), and an anti-DENV effector against four genetically distinct DENV serotypes (A. Buchman, Gamez, et al. 2020) (Figure 1.2K). Strategies against arboviruses such as ZIKV (A. Buchman et al. 2019) give insights into how modification approaches can be applied to re-emerging mosquito-borne pathogens (Figure 1.2L).

1.9 Comparing *Wolbachia* and transgenic approaches

While at the forefront of innovation for mosquito-borne disease control, *Wolbachia* and transgenic approaches have distinct advantages and limitations (Table 1.1). For example, in *Wolbachia* strategies, imperfect maternal transmission or CI loss can compromise replacement (Adekunle, Meehan, and McBryde 2019). Transgenic approaches have been successfully applied to many mosquito species, whereas success of *Wolbachia* transfection seems to be somewhat species-dependent (e.g., successful in *Ae. aegypti(McMeniman et al. 2009)*, but more difficult in *An. gambiae* (Chrostek and Gerth 2019)). Transgene-based approaches are highly flexible and optimizable, providing different levels of spatiotemporal spread, persistence, and many potentially novel traits.

Gene-editing tools enable precise genetic changes that permit creative control approaches (Bui et al. 2020; M. Li et al. 2020; Ming Li et al. 2017; Ming Li, Akbari, and White 2018). In contrast, current technologies do not allow *Wolbachia* engineering; thus, *Wolbachia*-based methods depend on the properties of the *Wolbachia* strains that are found in nature. *Wolbachia*

and transgenic approaches differ in their wild population-spreading abilities. For example, theoretical modeling suggests that some HGD systems can be established in a population within a year of the initial release (Ming Li et al. 2020; Sánchez C et al. 2020). Conversely, it may take *Wolbachia* 6 months to >2 years to become established (Hancock et al. 2016) and these rates can be impacted by temperature (Ross et al. 2017). Nevertheless, field releases of *Wolbachia*-infected mosquitoes demonstrate successful *Wolbachia* spread and effective pathogen blocking can be achieved (Aliota et al. 2016; Indriani et al. 2020).

Transgenic approaches can be tailored to specific pathogens through various mechanisms (RNAi, immune pathways, etc.). By contrast, although *Wolbachia* can target several pathogens, it can do so only through mechanisms dictated by this bacterium - which are not well understood. This mechanistic aspect is an important design consideration, as it influences the ability to target a pathogen at different developmental stages or multiple sites of its genome, which can be crucial for preventing or retarding emergence of pathogen resistance (Marshall et al. 2019; Dong, Simões, and Dimopoulos 2020). Still, natural *Wolbachia* strains are capable of blocking many recently emerging arboviruses (Dutra et al. 2016; Pereira et al. 2018), circumventing the need to produce additional transgenic tools that are tailored to each pathogen.

IIT requires the exclusive release of *Wolbachia*-infected male mosquitoes, which requires considerable effort. However, when combined with SIT (Zheng et al. 2019) or automated sex-sorting technology (Crawford et al. 2020), IIT approaches can be more efficient and cost-effective. Sex separation can be built into transgenic sterile-male approaches (Galizi et al. 2016; Kandul, Liu, Hsu, et al. 2020). Despite some differences in detail, all sterile-male methods offer excellent species specificity, spatiotemporal control, and reversibility. However, they all require much

higher release numbers than GD methods, in which the control agent or modification can propagate itself in the environment.

Some *Wolbachia* strains can block pathogen transmission, enabling release without sex-sorting, with the intent of *Wolbachia*-based population replacement (i.e., using the GD property of *Wolbachia*). Like engineered GDs, *Wolbachia* is able to quickly invade wild populations and reduce pathogen transmission (Ryan et al. 2019). Genetic control systems are intended to be transmitted only from parents to offspring ('vertical transmission'). The possibility of horizontal transmission to non-target species, followed by spread within that species, has been widely discussed (Cooper et al. 2019), but seems highly implausible. Some natural GDs, including transposons, transmit horizontally on evolutionary timescales; however, engineered GDs lack non-chromosomal intermediates facilitating interspecies transfer. Even then, only the most invasive drives can plausibly invade from an initial, rare transfer event (Almeida, de Almeida, and Carareto 2005).

Regulatory hurdles and public perception differ substantially between the two methods. Although mosquitoes transfected with *Wolbachia* are clearly modern biotechnological products, they have not encountered the same regulatory hurdles as transgenic approaches and go through different regulatory pathways (Chakradhar 2015) (e.g., *Wolbachia*-based approaches are considered 'veterinary chemical products' in Australia(Barro et al. 2011; DeBarro 2016)). This lack of regulatory clarity is an issue for genetically and non-genetically modified methods in many jurisdictions (Schwindenhammer 2020). Early engagement with communities, stakeholders, and the public has led to fewer public-relation barriers for *Wolbachia*-based approaches (Dickens et al. 2016). Both *Wolbachia* suppression (IIT) and modification approaches have been successfully trialed in several countries. The same is true for transgenic suppression approaches; the RIDL

approach successfully obtained regulatory and community approval in several countries, despite the more complex environment for Genetically Modified Organisms. Multiple trials in Brazil, Panama, and the Cayman Islands showed strong suppression of target *Aedes* mosquito populations. However, approval for the more recently developed transgene-based GD approaches appears to be lacking. Other programs, such as Target Malaria, have taken a meticulous, cautious approach towards approval for transgenic GD releases.

Finally, a remediation plan in case of failure is essential. Both approaches have the possibility of losing function (e.g., loss of transgene expression, or loss of CI or refractoriness in *Wolbachia* (Ross et al. 2019)) or being affected by an unintended consequence, shift in public opinion, or end of a trial period. Resistance alleles can limit transgene spread for GDs. However, innovative drive designs, such as reversal GDs that recall a problematic GD from the population (Vella et al. 2017; Wu, Luo, and Gao 2016; J. Champer, Buchman, and Akbari 2016), have been proposed to address this problem (J. Champer et al. 2018; Marshall et al. 2017; S. E. Champer et al. 2020). Anti-CRISPR proteins can theoretically be applied as 'natural brakes' to CRISPR-based HGDs, (Marino et al. 2020; Basgall et al. 2018) although proof-of-principle experiments are needed in mosquitoes. Ongoing field studies indicate that *Wolbachia* can remain at high infection frequency with strong pathogen blocking and CI abilities for up to 8 years from first invasion (Ryan et al. 2019). However, there is no easy way to remove *Wolbachia*-infected mosquitoes after release. One could release mosquitoes infected with a different *Wolbachia* strain and exploit bidirectional incompatibilities between the two strains, replacing the old strain with the new one (Joubert et al. 2016). Alternatively one could super-infect the old mosquito strain with an additional *Wolbachia* strain to generate a new strain that can spread into the already-invaded population. However, this scenario can lead to superinfection, where mosquitoes with multiple

Wolbachia strains can have incomplete maternal transmission or incompatible CI (Thomas H. Ant and Sinkins 2018). Release of wild-type mosquitoes to dilute the *Wolbachia* strain to sub-threshold levels is another potential remediation strategy; however, such wild-type releases would include large numbers of wild-type female mosquitoes capable of disease transmission and may be subideal. Finally, insecticide-based tools could be used for remediation in both cases, but the limitations of these approaches are well-established.

1.10 Concluding remarks and future perspectives

Wolbachia and transgene-based tools are innovative approaches that have revolutionized mosquito control. Immense progress has been made in genetically modified and *Wolbachia*-infected mosquitoes, leading to field trials around the world. Despite appreciable progress, knowledge gaps remain regarding *Wolbachia*-mosquito and *Wolbachia*-pathogen interactions. For example, not much is known about environment-host interactions, or how the host microbiome affects *Wolbachia* efficiency in mosquitoes (King et al. 2018). More work is needed to optimize CI and pathogen-blocking capabilities. Screening for temperature-insensitive *Wolbachia* strains is crucial to avoid CI loss (Ross et al. 2019). Identification and characterization of CI-inducing genes can pave the way for alternative control strategies. Likewise, more work on transgene-based strategies is required, including reducing the cost of transgene fitness, finding ideal target sites for GD insertion, and eliminating resistance-allele formation. For sterile-male-based suppression approaches, male mosquitoes must be released multiple times. Improvements in mass mosquito production, precise sex separation, and release technologies are crucial to make these approaches more sustainable and cost-efficient (Crawford et al. 2020).

Ethical and regulatory issues over GD use, including the role of public participation in GD development, informed consent, regulation, and associated risks, should be considered before implementation (Callies 2019; Schairer et al. 2019; Cheung et al. 2020). Discussions regarding who should regulate and assess the risks of GD technology are in process and may take years for a consensus. *Wolbachia* are naturally present in most insects, making this an easier technology for the public to accept. Several countries, including the United States, Singapore, Australia, China, Brazil, and Malaysia, have already released *Wolbachia*-infected mosquitoes on modest scales, with some observing reductions in local mosquito-borne disease transmission (Nazni et al. 2019; Crawford et al. 2020; Zheng et al. 2019; Ryan et al. 2019; Garcia et al. 2019; Servick 2016; hsuliyang 2016). Regardless, scientists should scrutinize all proposed technologies to fully understand their advantages and disadvantages. There is no single best solution for mosquito control, and different communities may prefer different approaches that suit their local needs. Therefore, the development of multiple approaches is crucial.

The prospect of controlling mosquito-borne diseases using innovative technologies is promising. With increasing public confidence, time, and progress, we will soon see these technologies used to tackle global health issues at scale.

1.11 Acknowledgements

This chapter, in full, is a reprint of the material that was submitted for publication. Guan-Hong Wang[1], Stephanie Gamez[1], Robyn Raban., John M. Marshall., Luke Alphey, Ming Li, Jason L. Rasgon., Omar S. Akbari. Combating mosquito-borne pathogens using genetic control technologies. (2020) Submitted to Nature Biotechnology [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

1.12 Figures

Figure 1.1: *Wolbachia* and transgene-based approaches for mosquito population suppression and population modification. (A) *Wolbachia* and transgene-based approaches for population suppression. *Wolbachia*-infected males can suppress mosquito populations through CI effects in the early embryo. To prevent fertile *Wolbachia*-infected females from escaping the sex-sorting step, an irradiation step is included to render them sterile. Using transgene-based approaches, mosquitoes can be engineered to induce lethality in the immature or adult stage of the life cycle. In suppression approaches, reducing the number of mosquitoes will reduce pathogen transmission. (B) *Wolbachia* and transgene-based approaches for population modification. Several studies have demonstrated the pathogen-blocking capabilities of *Wolbachia*. This feature can be used to modify mosquito populations for pathogen resistance. As *Wolbachia*-infected females have reproductive manipulation advantages (due to CI), pathogen blocking can spread throughout wild mosquito populations. In transgene-based approaches, strategies can be designed to inhibit replication of a specific pathogen through a desired mechanism (RNAi, over-expression of innate immune pathways, etc.). When linked to a gene drive, these strategies will spread throughout mosquito populations. Both *Wolbachia* and transgene-based approaches seek to maintain the mosquito population. Arrows represent mosquito releases. The multiple arrows in the *Wolbachia* IIT approach indicate that multiple releases are needed to achieve suppression. For simplicity, the SIT, pgSIT, RIDL, and fsRDIL approaches are mentioned as examples in panel A due to their requirement of multiple releases. These approaches do not utilize *Wolbachia*, despite being under this category in the figure. MAYV, mayaro virus, CHIKV, chikungunya virus; DENV, dengue virus; WNV, West Nile virus; ZIKV, Zika virus.

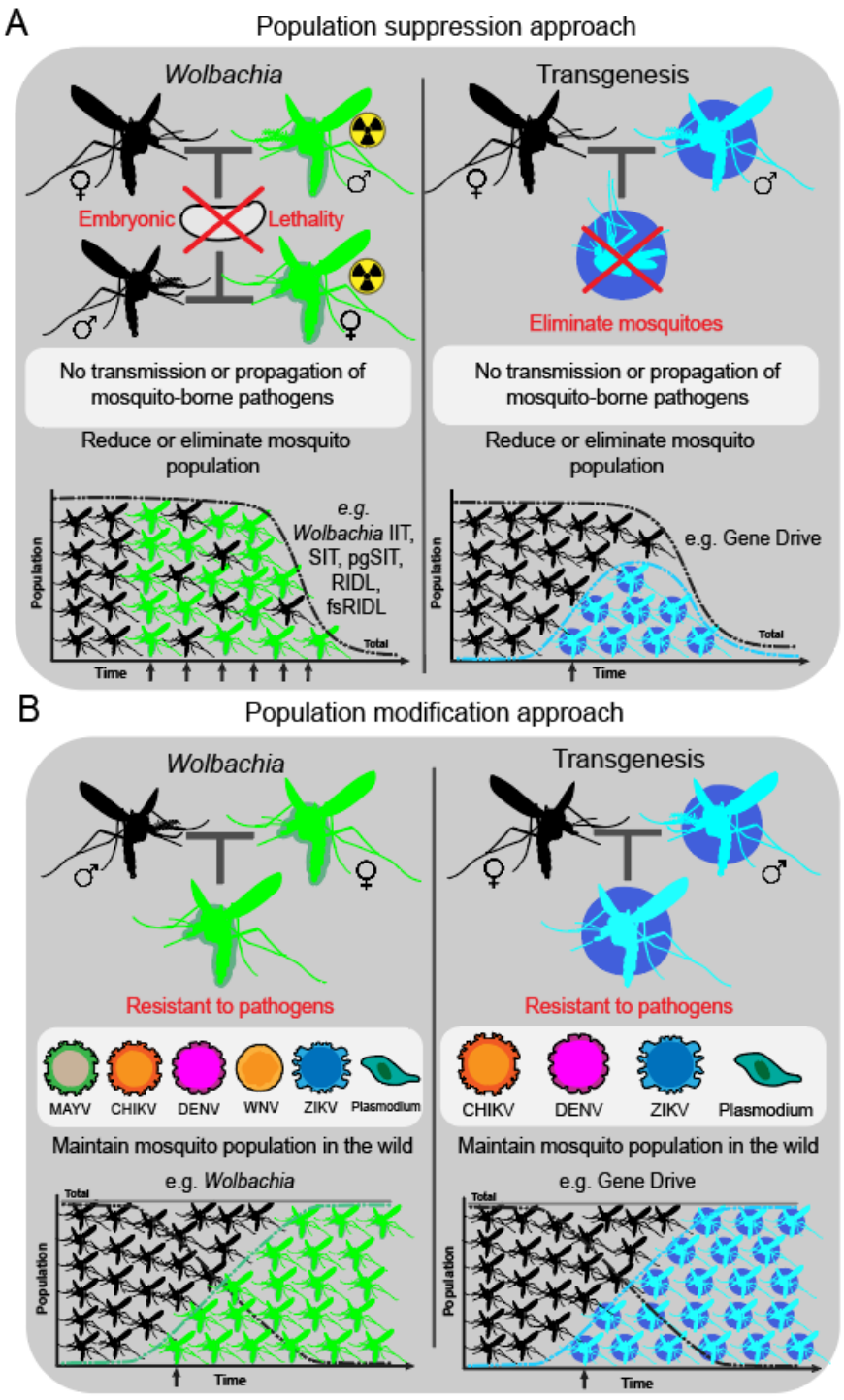
A
Population suppression approach
Wolbachia
Transgenesis
Embryonic
Lethality
Eliminate mosquitoes
No transmission or propagation of mosquito-borne pathogens
No transmission or propagation of mosquito-borne pathogens
Reduce or eliminate mosquito population
Reduce or eliminate mosquito population
e.g. Wolbachia IIT, SIT, pgSIT, RIDL, fsRIDL
e.g. Gene Drive
Population
Total
Time
B
Population modification approach
Wolbachia
Transgenesis
Resistant to pathogens
Resistant to pathogens
MAYV
CHIKV
DENV
WNV
ZIKV
Plasmodium
CHIKV
DENV
ZIKV
Plasmodium
Maintain mosquito population in the wild
Maintain mosquito population in the wild
e.g. Wolbachia
e.g. Gene Drive
Total
Population
Time

Figure 1.2: Examples of novel suppression and modification approaches in transgenic mosquitoes. Illustrations of recently developed population suppression approaches that utilize unique components to achieve mosquito suppression. (A) Gene drive (GD) suppression approach for *Anopheles* mosquitoes, which takes advantage of the sex determination pathway to produce fertile males and sterile females (Kyrou et al. 2018). (B) Sex-distorter GD programmed to home into *dsx* and express an endonuclease that shreds the X-chromosome (Simoni et al. 2020). High sex-bias ratios towards males enable a population crash after sufficient generations. (C) RIDL, a self-limiting approach, consists of a dominant lethal gene that utilizes modified components of the Tet-OFF operon system (Fu et al. 2010; Labbé et al. 2012). In the absence of tetracycline, transactivator (TtaV, green) binds to the operon sequence (orange) to induce toxic product expression in a tissue- and temporal-specific manner. High concentrations of toxic product will lead to lethality. (D) fsRIDL, a similar approach to RIDL, with added sex-specificity. A sex-specific intron ensures that TtaV protein will express only in flight muscles of females to prevent them from flying (Fu et al. 2010; Labbé et al. 2012). (E) Potential application of pgSIT in mosquitoes. Transgenic mosquitoes carrying components encoding Cas9 and several guide RNAs (gRNAs) targeting sex-determination genes will enable production of sterile male offspring (Kandul et al. 2019). (F) Self-limiting split drive (Ming Li et al. 2020). Separating both Cas9 and gRNA/GD element components enables a safe, noninvasive, self-limiting system. (G) Recoded GD prevents fitness load associated with disrupting two copies of *kh* gene (Adolfi et al. 2020). (H) Non-autonomous GD designed to have minimal components is used to produce an antimicrobial peptide in mosquito midgut to inhibit *Plasmodium* in these tissues (Hoermann et al. 2020). (I) Multistage effector transgenes with capacity to target several life stages of *Plasmodium* (Dong, Simões, and Dimopoulos 2020). Transgene containing five antimicrobial peptides is expressed after a blood meal. In another configuration, a single-chain antibody linked to an antimicrobial peptide was effective. (J) Transgenes produce microRNAs to induce the RNAi pathway of mosquitoes to target and inhibit dengue virus serotype 3 (DENV-3) and chikungunya virus (CHIKV) replication and transmission (Yen et al. 2018). (K) Anti-DENV transgene expresses an engineered single-chain antibody to confer resistance to four DENV serotypes (A. Buchman, Gamez, et al. 2020). (L) Anti-Zika virus (ZIKV) transgene uses eight synthetic small RNAs to induce the RNAi pathway against ZIKV (A. Buchman et al. 2019).

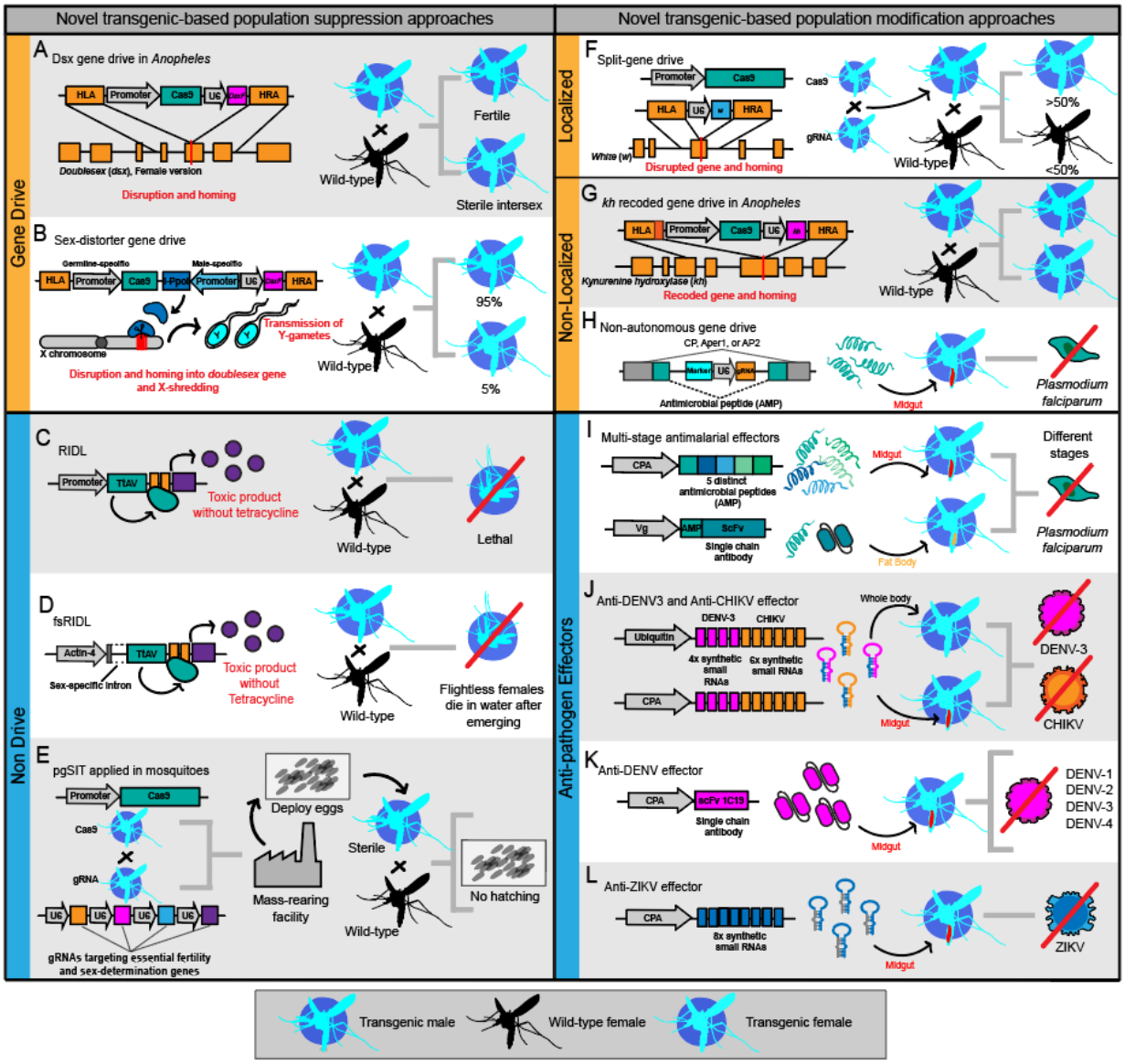
Novel transgenic-based population suppression approaches
Novel transgenic-based population modification approaches
Gene Drive
Non Drive
Localized
Non-Localized
Anti-pathogen Effectors
A Dsx gene drive in Anopheles
HLA
Promoter
Cas9
U6
Dsx
HRA
Doublesex (dsx), Female version
Disruption and homing
Wild-type
Fertile
Sterile intersex
B Sex-distorter gene drive
Germline-specific
Male-specific
Ppo
X chromosome
Transmission of Y-gametes
Disruption and homing into doublesex gene and X-shredding
95%
5%
C RIDL
Promoter
TtAV
Toxic product without tetracycline
Lethal
D fsRIDL
Actin-4
Sex-specific intron
Toxic product without Tetracycline
Flightless females die in water after emerging
E pgSIT applied in mosquitoes
Cas9
gRNA
U6
gRNAs targeting essential fertility and sex-determination genes
Deploy eggs
Mass-rearing facility
Sterile
No hatching
F Split-gene drive
HLA
U6
w
HRA
White (w)
Disrupted gene and homing
>50%
<50%
G kh recoded gene drive in Anopheles
kh
Kynurenine hydroxylase (kh)
Recoded gene and homing
H Non-autonomous gene drive
CP, Aper1, or AP2
Marker
gRNA
Antimicrobial peptide (AMP)
Midgut
Plasmodium falciparum
I Multi-stage antimalarial effectors
CPA
5 distinct antimicrobial peptides (AMP)
Vg
AMP
ScFv
Single chain antibody
Fat Body
Different stages
J Anti-DENV3 and Anti-CHIKV effector
Ubiquitin
DENV-3
CHIKV
4x synthetic small RNAs
6x synthetic small RNAs
Whole body
DENV-3
CHIKV
K Anti-DENV effector
scFv 1C19
DENV-1
DENV-2
DENV-3
DENV-4
L Anti-ZIKV effector
8x synthetic small RNAs
ZIKV
Transgenic male
Wild-type female
Transgenic female

Table 1.1: Comparison between *Wolbachia* and transgene-based approaches.

	Population modification			**Population suppression**			
Approach	*Wolbachia*	HGD	*ClvR*	IIT (*Wolbachia*)	HGD	RIDL	pgSIT
Proof-of- principle in mosquitoes?	Yes	Yes	No	Yes	Yes	Yes	In progress
Confinable?	Depends on fitness cost	Depends on GD type	Yes	Yes	Depends on GD type	Yes	Yes
Capacity to genetically engineer multiple strains	Not yet shown	Feasible	Feasible	Not yet shown	Feasible	Feasible	Feasible
Reversible?	No	Depends on GD type	Yes	Yes	Depends on GD type	Yes	Yes
Field releases or large cage studies	Australia(Ryan et al. 2019), Malaysia(Nazni et al. 2019), Indonesia(Tantowijoyo et al. 2020), Vietnam(Nguyen et al. 2015), Colombia(Aliota et al. 2016), Brazil(Servick 2016)	None	None	China(Zheng et al. 2019), USA(Crawford et al. 2020), Singapore(hsuliyang 2016)	Large cage assessments for a Sex-distorter(Facchinelli et al. 2019)	Brazil(Carvalho et al. 2015), Cayman Islands(Harris et al. 2011), Panama(Gorman et al. 2016)	None
Release frequency	Medium	Depends on design/ fitness	Low or high	High	Depends on design/ fitness	High	High
Mechanism of evolving resistance?	Temperature, host physiology, pathogen evolves resistance	Mutations in CRISPR machinery, NHEJ* events, natural polymorphisms at target site, effector gains nonfunctional mutations, pathogen evolves resistance	Mutations in CRISPR machinery, NHEJ events, natural polymorphisms at target site	Temperature, host physiology	Mutations in CRISPR machinery, natural polymorphisms at target site, NHEJ events	Second-site suppressor of zygotic lethality	Mutations in CRISPR machinery, present natural polymorphisms at target sites, NHEJ events

*NHEJ: Nonhomologous end-joining

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Chapter 2: Engineered resistance to Zika virus in transgenic *Ae. aegypti* expressing a polycistronic cluster of synthetic small RNAs

2.1 Abstract

Recent Zika virus (ZIKV) outbreaks have highlighted the necessity for development of novel vector control strategies to combat arboviral transmission, including genetic versions of the sterile insect technique, artificial infection with *Wolbachia* to reduce population size and/or vectoring competency, and gene drive based methods. Here, we describe the development of mosquitoes synthetically engineered to impede vector competence to ZIKV. We demonstrate that a polycistronic cluster of engineered synthetic small RNAs targeting ZIKV is expressed and fully processed in *Ae. aegypti*, ensuring the formation of mature synthetic small RNAs in the midgut where ZIKV resides in the early stages of infection. Critically, we demonstrate that engineered *Ae. aegypti* mosquitoes harboring the anti-ZIKV transgene have significantly reduced viral infection, dissemination, and transmission rates of ZIKV. Taken together, these compelling results provide a promising path forward for development of effective genetic-based ZIKV control strategies, which could potentially be extended to curtail other arboviruses.

2.2 Introduction

Since being introduced into the Americas, Zika virus (ZIKV) - a mosquito-borne flavivirus - has spread rapidly, causing hundreds of thousands of cases of ZIKV infection (Chitti, Prasad, and Saxena 2016). Although most cases remain asymptomatic, infection during pregnancy has been associated with severe congenital abnormalities and pregnancy loss, presenting an unprecedented health threat with long-term consequences ("Zika Virus and Complications: Questions and Answers" 2017). This prompted the World Health Organization to declare ZIKV a

Public Health Emergency of International Concern in 2016 ("Zika Virus and Complications: Questions and Answers" 2017; Chitti, Prasad, and Saxena 2016). Currently, there are no clinically approved vaccines to prevent ZIKV and no effective treatment options for infected individuals; thus, vector control remains essential in curtailing the ZIKV epidemic. Like dengue virus (DENV) and chikungunya virus (CHIKV), ZIKV is transmitted primarily by *Aedes* mosquitoes, which are expanding their habitable range due to urbanization, climate change, and global trade (Y.-J. S. Huang, Higgs, and Vanlandingham 2017). Current methods of vector control, including removal of standing water and use of insecticides, have not been entirely effective in the fight against the spread of *Aedes* mosquitoes (Y.-J. S. Huang, Higgs, and Vanlandingham 2017). Therefore, novel innovative vector control strategies, including those utilizing genetically engineered mosquitoes (Champer, Buchman, and Akbari 2016), are urgently needed to combat the spread of ZIKV and other *Aedes*-vectored diseases worldwide.

Employment of genetically modified (or otherwise altered) insects to manipulate disease-vectoring populations was first proposed decades ago (Curtis 1968), and due in part to enabling technological advances, has garnered increased interest in recent years (Champer, Buchman, and Akbari 2016; Kandul et al. 2018). In fact, several strategies for genetic-based vector control are currently being utilized in the field. For example, the RIDL (Release of Insects carrying a Dominant Lethal) system (Alphey et al. 2013) - a genetic-based Sterile Insect Technique (SIT)-like system - has recently been shown to be effective in reducing wild insect populations (Harris et al. 2012). Open field release trials of these genetically modified mosquitoes have been conducted in several countries, including Cayman Islands, Malaysia, Brazil, and are currently being considered for use in India and the USA (Carvalho et al. 2015; Pollack 2016; Doyle 2016). In addition to genetic-based vector control strategies, mosquitoes harboring artificially acquired

strains of *Wolbachia* can also be used to either reduce total insect populations (Bourtzis et al. 2014) or to render insect populations less competent vectors of certain viruses, including ZIKV (Dutra et al. 2016), and this technique has also been trialed in multiple countries to reduce impact of mosquito-borne diseases (Waltz 2017; Marris 2017; Callaway 2016). (Although the accumulating evidence that *Wolbachia* can enhance certain flavivirus infections (Dodson et al. 2014; Amuzu et al. 2018; King et al. 2018) may lead do reevaluation of this technique.) Nevertheless, while current approaches can be effective, they require inundative releases of large numbers of insects, which can be laborious and expensive and can impede scalability and worldwide adoption.

Another category of genetic-based vector control involves an engineered gene drive system that can force inheritance in a super-Mendelian fashion, enabling it to increase itself - and any linked "cargo" genes - in frequency with each generation even without conferring fitness advantages to its host (Burt 2014; Champer, Buchman, and Akbari 2016). Such a method could be used to disseminate desirable "cargo" genes, such as pathogen resistance, rapidly through wild disease-transmitting populations, modifying vector populations to be disease-refractory (Sinkins and Gould 2006). While significant efforts are currently underway to develop engineered drive systems (Gantz et al. 2015; Hammond et al. 2016; Akbari et al. 2014; Chen et al. 2007), others are focused on creation of "cargo" genes that may be spread by the drive systems, and several studies have reported on the successful development of pathogen resistance "cargo" genes in *Ae. aegypti* (Jupatanakul et al. 2017; A. W. Franz et al. 2006; Mathur et al. 2010).

To date, however, no anti-ZIKV refractory "cargo" genes in any mosquito have been developed. To fill this void, here we describe the generation of a synthetically engineered ZIKV resistance transgene comprising a polycistronic cluster of ZIKV-targeting synthetic small RNAs. We demonstrate that *Ae. aegypti* mosquitoes harboring this anti-ZIKV transgene express and fully

process the ZIKV-targeting synthetic small RNAs in the midgut and consequently have significantly reduced viral infection, dissemination, and transmission rates of ZIKV. Specifically, we demonstrate that mosquitoes homozygous for the anti-ZIKV transgene are fully resistant to ZIKV infection and are unable to transmit the virus. In contrast, we determine that a minority of heterozygotes for the anti-ZIKV transgene can become infected with ZIKV following exposure. However, these heterozygotes become infected at significantly lower rates than wildtypes (WT), and those susceptible to infection have roughly three orders of magnitude lower viral titres in their saliva, suggesting a significantly reduced possibility of viral transmission. This is supported by our finding that heterozygous anti-ZIKV mosquitoes are almost entirely incapable of *in vivo* ZIKV transmission in a sensitive *Stat1-/-* mouse model. Moreover, when compared to *Wolbachia w*Mel positive mosquitoes, previously shown to have reduced ZIKV vectoring competency (Dutra et al. 2016), the anti-ZIKV mosquitoes perform significantly better in the ZIKV challenge assays. Taken together, these compelling results provide a promising path forward for development of effective ZIKV control - and possibly control of other clinically significant arboviruses - using genetically engineered mosquitoes.

2.3 Methods

2.3.1 Synthetic anti-ZIKV small RNAs design and construction

The *Drosophila melanogaster* miR6.1 stem-loop, which has been previously validated in *D. melanogaster* (Akbari et al. 2014), was modified to target eight unique sites in the ZIKV polyprotein region as previously described (Chen et al. 2007). The eight target sites corresponded to regions of capsid (C), membrane precursor (prM), and envelope (E) structural genes, RNA-directed RNA polymerase NS5 (which contained three target sites), and non-structural proteins

NS1 and NS2A, of ZIKV strain H/PF/2013 (GenBank: KJ776791.2) (Baronti et al. 2014). These sites were highly conserved in ZIKV strain FSS13025 (Cambodia 2010, Genbank KU955593) (Ladner et al. 2016) and in ZIKV strain PRVABC59 (isolated from US traveller to Puerto Rico in 2015, GenBank KU501215) (Figure S2.1). To generate miR6.1 stem-loop backbones that create mature synthetic small RNAs complementary to each of these target sites, pairs of primers were annealed and products were utilized for two subsequent rounds of PCR and cloned into the pFusA backbone (from the Golden Gate TALEN and TAL Effector Kit 2.0, Addgene #1000000024) in sets of four using Golden Gate assembly (Engler, Kandzia, and Marillonnet 2008) to generate plasmids OA959A and OA959B. Assembled small RNAs were then digested with either PmeI/BglII (vector OA959A) or with BamHI/PacI (vector OA959B) and were subcloned into a PacI/PmeI-digested final vector OA959C (the anti-ZIKV transgene). The ZIKV target sequences and sequences of primers used in the small RNA cloning are listed in Table S2.5.

2.3.2 Plasmid assembly

To generate vector OA959C (the anti-ZIKV transgene), several components were cloned into the *piggyBac* plasmid pBac[3xP3-DsRed] (Li, Bui, Yang, Bowman, et al. 2017) using Gibson assembly/EA cloning (Gibson et al. 2009). First, a *Drosophila* codon optimized tdTomato marker was amplified with primers 959C.10A and 959C.10B from a gene synthesized vector (GenScript, Piscataway, NJ) and cloned into a XhoI/FseI digested pBac[3xP3-DsRed] backbone using EA cloning. The resulting plasmid was digested with AscI, and the following components were cloned in via EA cloning: the predicted *Aedes aegypti* carboxypeptidase promoter (Moreira et al. 2000) amplified from *Ae. aegypti* genomic DNA using primers 959C.11A and 959C.11B, a GFP sequence amplified from vector pMos[3xP3-eGFP] (Kokoza et al. 2001) with primers 959C.12A

and 959C.12B, and a 677 bp p10 3' untranslated region (UTR) amplified with primers 959C.13A and 959C.13B from vector pJFRC81-10XUAS-IVS-Syn21-GFP-p10 (Addgene plasmid #36432). Assembled small RNA fourmers were then subcloned into final plasmid OA959C using PacI and PmeI using traditional cloning. All primer sequences are listed in Table S2.6. Complete annotated plasmid sequence and DNA is available via Addgene (plasmid #104968).

2.3.3 Generation of transgenic mosquitoes

Germline transformations were carried out largely as described (Li, Bui, Yang, White, et al. 2017). Briefly, 0-1 hr old Higgs and Liverpool strain *Ae. aegypti* pre-blastoderm embryos were injected with a mixture of vector OA959C (200 ng/ul) and a source of *piggyBac* transposase (200 ng/ul) (Kokoza et al. 2001); the injected embryos were hatched in deoxygenated H_2O. A total of 52 surviving Higgs adult males and 64 surviving Higgs adult females, and 61 surviving adult Liverpool males and 75 surviving adult Liverpool females, respectively, were recovered after the injection. Higgs adults were assigned to 35 pools and Liverpool adults were assigned to 39 pools, and outcrossed to Higgs or Liverpool adults, respectively, of the opposite sex in cages. Larvae were fed ground fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany) and adults were fed with 0.3M aqueous sucrose. Adult females were blood fed three to five days after eclosion using anesthetized mice. All animals were handled in accordance with the guide for the care and use of laboratory animals as recommended by the National Institutes of Health and supervised by the local Institutional Animal Care and Use Committee (IACUC). A total of 8,189 Higgs and 10,949 Liverpool G_1s were screened. Larvae with positive fluorescent signals (3xp3-tdTomato) were selected under the fluorescent stereomicroscope (Leica M165FC) and were crossed to establish stable transgenic lines. Four independent lines (termed TZIKV-A, B, and D recovered

from Liverpool G_1s, and TZIKV-C recovered from Higgs G_1s) with the strongest fluorescence expression patterns were selected for further characterization. To determine whether these lines represented single chromosomal insertions, we backcrossed single individuals from each of the lines for four generations to wild-type stock, and measured the Mendelian transmission ratios in each generation; in all cases, we observed a 50% transmission ratio, indicating insertion into single chromosomes. For one of the four lines (TZIKV-C), transgenic mosquitoes were inbred for at least 12 generations to generate a homozygous stock. Mosquito husbandry was performed under standard conditions as previously described (Akbari et al. 2013).

2.3.4 Characterization of transgene genomic insertion sites

To characterize the insertion site of vector OA959C in transgenic mosquitoes, we adapted a previously described inverse polymerase chain reaction (iPCR) protocol (A. M. Huang, Rehm, and Rubin 2009) as follows. Genomic DNA (gDNA) was extracted from 10 transgenic *Ae. aegypti* fourth instar larvae of each line using the DNAeasy Blood & Tissue Kit (Qiagen #69504) per the manufacturer's protocol. Two separate restriction digests were performed on diluted gDNA to characterize the 5' and 3' ends of the insertion using Sau3AI (5' reaction) or HinP1I (3' reaction) restriction enzymes. A ligation step using NEB T4 DNA Ligase (NEB #M0202S) was performed on the restriction digest products to circularize digested gDNA fragments, and two subsequent rounds of PCR were carried out per ligation using corresponding *piggyBac* primers listed in SI Appendix Table S7. Final PCR products were cleaned up using the MinElute PCR Purification Kit (Qiagen #28004) in accordance with the manufacturer's protocol, and sequenced via Sanger sequencing (Source BioScience, Nottingham, UK). To confirm transgene insertion locus and orientation via PCR, primers were designed based on iPCR mapped genomic regions and used in

tandem with *piggyBac* primers based on their location as listed in Table S2.7. Sequencing data was then blasted to the AaegL5.0 reference genome (NCBI). An alignment of the sequencing data was carried out with SeqManPro (DNASTAR, Madison, WI) to determine orientation of the transgene insertion site. Analysis of the sequencing data indicated that the insertion sites were on chromosome 2 (at approximate position 167,899,561) for line TZIKV-A, on chromosome 3 (at approximate position 402,525,313) for line TZIKV-B, on chromosome 3 (at approximate position 173,647,938) for line TZIKV-C, and on chromosome 1 (at approximate position 228,972,549) for line TZIKV-D. These insertion locations were also confirmed by PCR and sequencing performed on genomic DNA from the transgenic mosquitoes.

2.3.5 Small RNA extraction, isolation, sequencing, and bioinformatics

Total RNA was extracted from midguts of 30 ZIKV-C transgenic and WT (Higgs strain) non-blood-fed adult females as well as midguts of 30 ZIKV-C transgenic and WT (Higgs strain) adult females 24 hours post blood-feeding using the Ambion mirVana mRNA Isolation Kit (ThermoFisher Scientific #AM1560). Following extraction, RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific #AM2238). The quality of RNA was assessed using RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513) and a NanoDrop 1000 UV–vis spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE). Small RNA was then extracted and prepared for sequencing with QIAseq miRNA Library Kit (Qiagen #331502). Libraries were quantified with Qubit dsDNA HS Kit (ThermoFisher Scientific #Q32854) and High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies #5067- 4626) and sequenced on Illumina HiSeq2500 in single read mode with the read length of 75 nt following manufacturer's instructions. After adapter trimming and UMI extraction, reads were aligned to

mature *Ae. aegypti* miRNAs downloaded from miRBase (release 22, (Kozomara and Griffiths-Jones 2014)) and to each synthetic small RNA's passenger, loop, and guide sequences using bowtie2 in 'very-sensitive-local' mode. (We assumed, based on the design of the synthetic small RNAs, that they are processed as miRNAs; however, it remains possible that they are instead processed as endogenous small RNAs (esiRNA) or some other small RNA species.) Custom Perl scripts were used to quantify the number of reads that mapped to each target. 5 out of 8 target sites were reliably detected at TPM values between 2 and 91. Sites 3, 5 and 7 were not detected above background in either of the transgenic samples (Table S2.1). Correlation coefficients of TPM values between WT and transgenic animals were calculated in R(Team 2014). Differential expression analysis was performed with R package DESeq2 using two factor design (design= ~ feeding + genotype). TPM values and MA plots were generated with R package ggplot2 (Figure S2.3). Quantification data are shown in Table S2.1. All sequencing data can be accessed at NCBI SRA (accession ID: SRP150144; BioProject ID: PRJNA475410).

2.3.6 RT-PCR confirmation of anti-ZIKV transgene expression

To assay synthetic small RNA expression in mosquitoes, total RNA was separately extracted from 50 dissected midguts and 6 carcasses (midguts and heads removed) of Higgs WT and ZIKV-C non blood fed females, as well as 30 dissected midguts and 6 carcasses (midguts and heads removed) of Higgs WT and ZIKV-C females 24 hours post blood-feeding using the Ambion mirVana mRNA Isolation Kit (ThermoFisher Scientific #AM1560). Following extraction, total RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific #AM2238). RNA was then converted to cDNA using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific #K1631) using a mix of oligo(dT)$_{18}$ and random hexamer primers. PCR was then

performed on the resulting cDNA using standard procedures. To confirm presence of synthetic small RNA transcripts, primers 959.S7 and 959.S8 were used to amplify a fragment from the 5'UTR region of the carboxypeptidase A promoter (downstream of the transcription start site) to the loop-guide strand region of small RNA 1. As a positive control, primers 959.S10 and 959.S11 were used to amplify a short sequence of the *Actin1* gene (AAEL011197)[(Dzaki et al. 2017)]. Expression of the anti-ZIKV transgene transcript was observed in both TZIKV-C midgut and carcass tissues regardless of mosquito blood meal state, but was completely absent in Higgs WT mosquito tissues (Figure S2.4), while *Actin1* positive control transcripts were present in all samples. PCR products were sequenced to confirm product identity. All primer sequences are listed in Table S2.7.

2.3.7 ZIKV infection of mosquitoes, virus determination and longevity

All experiments were performed under biosafety level 3 (BSL-3) conditions in the insectary at the Australian Animal Health Laboratory. Insectary conditions were maintained at 27.5℃ and 70% in relative humidity with a 12hr light/dark cycle. ZIKV strain FSS13025 (Cambodia 2010, Genbank KU955593) (Ladner et al. 2016) or PRVABC59 (Puerto Rico 2015, GenBank KU501215) were used for viral challenge experiments. Both belong to the Asian/Pacific/American clade and were passaged once in C6/36 cells and twice in Vero cells before using for mosquito infections. WT (Higgs strain for TZIKV-C experiments, Liverpool strain for TZIKV-A, B, and D experiments) and transgenic (confirmed by red fluorescence in the eye) mosquitoes were infected with ZIKV as previously described (Duchemin et al. 2017). Briefly, female mosquitoes were challenged with a chicken blood meal spiked with ZIKV ($TCID_{50}$ 10^6/mL) through chicken skin membrane feeding. Blood-fed female mosquitoes were sorted and

maintained at standard conditions in an environmental cabinet with sugar *ad libitum*. For infection rate and virus titer, mosquito midguts were collected at 4 dpi. For dissemination and transmission rate, mosquito saliva, midguts, and carcasses were collected at 14 dpi. Mosquito saliva was used to determine viral titers using $TCID_{50}$ assay on Vero cells. Midguts and carcasses were used to determine presence of viral RNA using RT-qPCR against ZIKV NS5 (Duchemin et al. 2017) (Table S2.7). Mosquito viral challenge, processing, saliva testing, and molecular analyses of infection and dissemination were carried out as previously described (Duchemin et al. 2017). ZIKV infection rate was defined by the number of midguts (4 dpi) found positive for viral nucleic acid over tested midguts. Similarly, the dissemination rate was calculated by the number of carcasses (14 dpi) testing ZIKV positive by qPCR. Transmission rate was defined by the number of $TCID_{50}$ positive saliva samples over the number tested. For each experiment, data from three replicates was pooled. The average $TCID_{50}$ values were compared by two-tailed unpaired t test. To measure fitness after infection, blood-fed ZIKV-infected females were quickly sorted out after CO_2 anaesthesia and housed in waxed cardboard cup 250 ml containers with a maximum of 25 mosquitoes. Mosquitoes were maintained at standard conditions for 14 days with 10% sugar solution *ad libitum*. Dead mosquitoes were counted daily. Females surviving at day 14 were marked as censored (status=0) in the database for survival analysis, which was performed using the GraphPad Prism software (GraphPad Software, La Jolla California, USA). The Mantel-Cox test was used to compare the survival of infected mosquitoes at 14 dpi.

2.3.8 Generation of *w*Mel *Wolbachia* line and infection assay

Eggs of *Ae. aegypti* infected with the *Wolbachia* strain *w*Mel were obtained from the World Mosquito Program (Prof. Scott O'Neill, Monash University). Higgs mosquitoes infected with

*w*Mel were generated by crossing *w*Mel+ females with males from the Higgs line, and the resulting offsprings were used for ZIKV infections experiments. At the end of the experiment, *Wolbachia* infection status of these mosquitoes was tested using PCR with primers specific for *w*Mel detection (Joubert et al. 2016) (Table S2.7). The PCRs indicated presence of *w*Mel in >90% of mosquitoes, and only results from these positive mosquitoes were used for further analysis.

2.3.9 Mouse transmission assays

All experiments were performed under biosafety level 3 (BSL-3) conditions in the insectary at NHRI. Insectary conditions were maintained at 29°C and 80% relative humidity with a 12 hr light/dark cycle, and mosquitoes were maintained as previously described (Kuo et al. 2018). For experimental assays, transgenic anti-TZIKV-C mosquitoes were outcrossed to WT (Higgs strain) for a generation to obtain heterozygotes. Non-transgenic sibling mosquitoes from the above cross were used as Higgs WT controls. ZIKV strain PRVABC59 (Puerto Rico 2015, GenBank KU501215) was used for viral challenge experiments. It was obtained from the Taiwan Center for Disease Control, and maintained/amplified as previously described (Kuo et al. 2018). For direct ZIKV infection, 7–10 day-old female TZIKV-C and Higgs WT mosquitoes were inoculated with 200 plaque forming units (pfu) of ZIKV by thoracic injection as previously described (Kuo et al. 2018) and maintained under standard housing conditions for 7 days prior to their use in assays. Infection via artificial membrane blood feeding was carried out as described above, and infected mosquitoes were then maintained under standard conditions for 14 days prior to their use in transmission assays. Viral titers were measured at 7 dpi (for thoracic injection infections) or 14 dpi (for membrane blood feeding infection) by plaque assay as previously described (Hsu et al. 2015; Kuo et al. 2018). Briefly, $2x10^5$ cells/well of Vero cells (a kind gift from Dr. Guann-Yi Yu)

were incubated for one day (in serum-free 1xDMEM medium (HyClone, SH30022), at 37℃) before being infected with ZIKV. At two hours post infection, unbound virus particles were removed, and cells were gently washed by PBS and overlaid with 3 ml of 1xDMEM medium containing 2% FBS (Gibco, 16000044), 10 mM HEPES, 10nM sodium pyruvate, 2mM L-Glutamine (Gibco, 25030081), 1x Penicillin-Streptomycin (Gibco, 15140122), and 1% Methyl cellulose (Sigma, M0512-250G). The infected cells were then incubated at 37℃ and 5% CO_2 for 4 days until plaque formation. Cells were fixed and stained with 0.5mL crystal violet/methanol mixed solution (ASK®Gram Stain Reagent) for 2 hours, and washed with H_2O. Number of plaques was then calculated, and viral titers were determined as plaque forming units per mosquito and were compared by one-way ANOVA.

All mouse-related experiments were conducted in compliance with the guidelines of the Laboratory Animal Center of NHRI. The animal protocol (NHRI-IACUC-105111) was approved by the Institutional Animal Care and Use Committee of NHRI, according to the Guide for the Care and Use of Laboratory Animals (NRC 2011). Management of animal experiments and animal care and use practices of NHRI have been accredited by the AAALAC International. *Stat1*-/- (C57BL/6 background) mice were provided by Dr. Guann-Yi Yu (NTU, Taiwan). Both male and female mice between the ages of 11-12 weeks were used in the study.

Mosquito-mediated ZIKV mouse infections were carried out as previously described (Hsu et al. 2015; Kuo et al. 2018). Briefly, mice were anesthetized with Ketalar (100 mg/Kg, Pfizer, New York, NY) via intraperitoneal injection, and their ventral surfaces were shaved. Then, mice were placed on top of a polyester mesh covering a mosquito-housing cage that permitted female mosquitoes to take a blood meal. Female mosquitoes were starved for 10h before they were allowed to take blood meals from mice, and each mouse was fed on by 6–11 mosquitoes. Mouse

body weight and mortality were then recorded for 6-30 days. Mouse weights were compared by the Mann Whitney test to evaluate for significant weight loss.

2.3.10 Fitness assessment and conditions

To determine if the anti-ZIKV transgene confers a fitness cost, several fitness parameters were evaluated in Higgs WT and TZIKV-C mosquitoes. For these experiments, homozygous TZIKV-C mosquito stock obtained after 12 generations of inbreeding (see above) and the Higgs WT stock utilized to obtain transgenic lines were used. Evaluation of all experimental and control replicates were performed simultaneously. Insectary conditions were maintained at 28℃ and 70-80% in relative humidity with a 12hr light/dark cycle. To assess larval to pupal development time, eggs were vacuum hatched and larvae were distributed into pans (50 larvae per pan) containing 2.5L of ddH_2O and 0.6mL of fish food slurry. To determine the development time of TZIKV-C and Higgs WT control mosquitoes, 4th instar larvae were sorted according to fluorescence phenotype and reared until pupation. Pupae were collected and counted every day until no pupae were left. To assess female fertility and fecundity, 90 TZIKV-C or Higgs WT females were mated to 20 Higgs WT males in a cage. After four days, females were blood fed and individually transferred into plastic vials filled with water and lined with egg paper. After three days, egg papers were collected, and eggs were counted and vacuum hatched in 9-ounce plastic cups. Starting on the fourth day, larvae were counted every day until no larvae were present. Female fecundity refers to the number of eggs laid per female, and fertility reflects the number of eggs hatching to produce larvae. To measure male mating success, fecundity, and fertility, one TZIKV-C or Higgs WT male was mated to five Higgs WT females in a single cup filled with water and lined with egg paper. Three days post blood meal, cups were checked for the presence of eggs, which were collected,

counted, and hatched. Hatched larvae were then counted every day until no larvae were present. Male mating success was calculated as the percentage of single male outcrosses that produced larvae. Fecundity was measured as the number of eggs laid per cup; fertility was determined by the number of hatching larvae in each cup. To assess wing length as a proxy for body size, images of TZIKV-C and Higgs WT mosquito wings were taken with a Leica M165 FC microscope (Leica Microsystems). Wing length measurements were done by using the measurement tool on the Leica Application Suite X, measuring from the axial incision to the intersection of the R 4+5 margin. Finally, to assess mosquito longevity, equal numbers of male and female TZIKV-C or Higgs WT mosquitoes were placed in medium sized cages (in triplicate). Mosquitoes that died were counted and removed daily until all mosquitoes had died. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla California, USA). Means were compared using unpaired t tests with Welch's correction except for male mating success (no Welch's correction). Analyses of mosquito survivorship utilized the Mantel-Cox test. *P*-values>0.05 were considered non-significant.

2.3.11 Confirmation of transgene zygosity

To molecularly confirm zygosity of transgenic mosquitoes, mosquito heads were homogenised using bead-beater for DNA extraction in 30 ul extraction buffer (1x Tris-EDTA, 0.1M EDTA, 1M NaCl and 2.5 uM proteinase K), and incubated at 56°C for 5 minutes and then at 98°C for 5 minutes. PCR was then performed on each line to detect the presence of the transgene by pairing a *piggyBac* primer with a genomic primer as follows: primers 1018.S46 and 991.5R2 for TZIK-A, 1018.S26 and 991.3F2 for TZIK-B, 1018.S8 and 991.5R1 for TZIK-C, and 1018.S50 and 991.3F2 for TZIK-D (Table S2.7). To determine zygosity, we amplified the WT locus of each

transgenic line using corresponding forward and reverse primers listed in SI Appendix Table S7. WT mosquitoes (Higgs strain for TZIKV-C assays, Liverpool for TZIKV-A, B, and D assays) served as controls to ensure that the WT locus was successfully amplified in each genetic background. A PCR kit (ThermoFisher Scientific #F553S) with 57°C annealing temperature and standard protocols was used for all PCRs.

2.3.12 Data availability statement

All sequencing data associated with this study are available from NCBI sequence read archive (SRA) accession ID: SRP150144; BioProject ID: PRJNA475410. Complete annotated plasmid sequence and DNA is publically available at Addgene (plasmid #104968). Transgenic mosquitoes will be made available by the corresponding author upon request.

2.4 Results

2.4.1 Engineering ZIKV-resistant mosquitoes

To generate a "cargo" gene that can confer resistance to ZIKV, we implemented a synthetic small RNA-based approach, since such an approach has been previously demonstrated to generate virus-resistance phenotypes in a number of contexts (e.g., (Niu et al. 2006; Saha et al. 2016; Xie et al. 2013)), including mosquitoes (Yen et al. 2018). We engineered a *piggyBac* vector comprising a polycistronic cluster of eight ZIKV-targeting miRNA-like synthetic small RNAs (the anti-ZIKV transgene) under control of the *Ae. aegypti* carboxypeptidase (CP) promoter (Moreira et al. 2000) to drive expression of the synthetic small RNAs in female midguts following a blood meal (Figure 2.1A). To ensure effective viral suppression and evolutionary stability, we designed each of the eight synthetic small RNAs to target 6/10 conserved protein-coding genes of French Polynesia

ZIKV strain H/PF/2013 (GenBank: KJ776791.2) (Baronti et al. 2014), including all three structural genes (capsid (C), membrane precursor (prM), envelope (E)), and three non-structural genes (NS1, NS2A, NS5). Each of these genes was targeted by a single synthetic small RNA, except for the RNA-dependent RNA polymerase NS5, which was targeted by three small RNAs due to its importance for the replication of the flaviviral RNA genome (Figure 2.1B, Figure S2.1). The engineered anti-ZIKV transgene (termed plasmid OA959C) also contained the eye-specific 3xP3 promoter (Berghammer, Klingler, and Wimmer 1999) driving expression of tdTomato as a transgenesis marker (Figure 2.1A).

Following embryonic microinjection, multiple transgenic lines were identified ($n > 6$), and four independent lines with strong expression of tdTomato fluorescence in the eyes (termed TZIKV-A, B, C, and D) were selected for further characterization (see Figure 2.1C, 2.1D for fluorescence in TZIKV-C). To verify the transgene insertion sites, we performed inverse PCR (iPCR) on genomic DNA extracted from transgenic mosquitoes of all four independent strains. iPCR analysis indicated that insertion sites were on chromosome 2 (at approximate position 167,899,561) for line TZIKV-A, on chromosome 3 (at approximate position 402,525,313) for line TZIKV-B, on chromosome 3 (at approximate position 173,647,983) for line TZIKV-C, and on chromosome 1 (at approximate position 228,972,549) for line TZIKV-D when aligned to the AaegL5 assembly (GenBank assembly accession: GCA_002204515.1) (B. J. Matthews n.d.) . To avoid any bias due to position effect variegation (PEV) stemming from transgene insertion sites, all four lines were screened for midgut infection status at 4 days post-infection (dpi), and results showed that all 4 lines had significant reduction in midgut infection rate and viral titres compared with Higgs or Liverpool WT mosquitoes (Figure S2.2). Given that there was no significant difference in ZIKV reduction in midgut infection between the four lines (TZIKV-A, B, C, and D),

the line exhibiting the strongest antiviral phenotype (TZIKV-C) was selected for further comprehensive characterization (Figure S2.2).

2.4.2 Molecular analysis of synthetic small RNA expression and processing

To confirm expression and processing of the ZIKV-targeting synthetic small RNAs in TZIKV-C, we deep-sequenced small RNA populations from dissected midgut tissues isolated from blood fed and non blood fed female mosquitoes using an Illumina platform. We detected expression of the non-guide (Meijer, Smith, and Bushell 2014) and mature small RNA guide strands of 5 out of 8 anti-ZIKV-targeting synthetic small RNAs (small RNAs 1,2,4,6, and 8) with TPM (transcripts per million) values for mature small RNA guide strands ranging from 2 to 91, 25.7 on average, indicating that these synthetic small RNAs are efficiently expressed and processed (Figure S2.3, Table S2.1). Importantly, no anti-ZIKV-targeting small RNAs (>1 read) were identified in small RNA populations derived from Higgs WT *Ae. aegypti* (Table S2.1).

We also performed RT-PCR assays on dissected midgut tissues and midgut-free carcasses from blood fed and non blood fed female mosquitoes to determine whether synthetic small RNA expression was confined to the midgut. Contrary to previously published reports (Moreira et al. 2000; A. W. E. Franz et al. 2011), we found that the carboxypeptidase promoter drove detectable expression of the synthetic small RNAs in tissues other than the midgut, and that expression occurred even without a blood meal (Figure S2.4), suggesting that expression of the anti-ZIKV transgene may be strongly affected by its genomic insertion position. However, importantly, no anti-ZIKV-targeting small RNAs were detected in Higgs WT *Ae. aegypti* (Figure S2.4). Taken together, these results demonstrate that the anti-ZIKV transgene is stably integrated into the

mosquito genome and most of the ZIKV-targeting synthetic small RNAs are expressed and processed in an appropriate context (including in the midgut) for ZIKV suppression.

2.4.3 Engineered mosquitoes are refractory to multiple ZIKV strains

To characterize the functional significance of ZIKV-targeting synthetic small RNA expression and processing on vector competence, adult female mosquitoes (Higgs WT control and TZIKV-C) were infected with ZIKV (FSS13025, Cambodia 2010 strain, GenBank JN860885) via membrane blood-feeding (Ladner et al. 2016). For these experiments we used the Cambodia ZIKV strain, which is from the Asian ZIKV lineage and in close phylogenetic proximity to the French Polynesia ZIKV strain against which the small RNAs were designed (Gubler, Vasilakis, and Musso 2017). Importantly, seven out of eight of the ZIKV-targeting synthetic small RNA target sites are 100% conserved between the Cambodia ZIKV strain and the French Polynesia strain, allowing either strain to be used for the ZIKV challenges (Figure S2.1). At 4 days post-infection (dpi), midguts from blood-fed mosquitoes were dissected and ZIKV RNA copies were measured using real-time RT-qPCR. Results from three biological replicates revealed that none of the TZIKV-C mosquitoes homozygous for the transgene (n=32) were positive for ZIKV infection in the midguts. ZIKV infection was detected in 87.5% (28/32) of the TZIKV-C mosquitoes that were heterozygous for the transgene; however, these mosquitoes had significantly ($p<0.001$) lower viral RNA levels (~2 logs) than Higgs WT (Figure 2.2A, Table S2.2).

To assay for viral dissemination, total RNA was collected from whole TZIKV-C mosquito carcasses and dissected midguts from both homozygous and heterozygous transgenic mosquitoes at 14 dpi. The results from three biological replicates indicated that none of the homozygous TZIKV-C mosquitoes (n=46) were positive for viral replication (dissemination) in either the

midgut or the carcass. ZIKV prevalence was detected in 74.4% (29/39) of heterozygous TZIKV-C mosquitoes in both the carcass and midgut; however, they had significantly ($p<0.001$) lower levels of viral RNA (~3 logs) compared to Higgs WT (Figure 2.2B-C, Table S2.2). Finally, to determine viral transmission, saliva from individual mosquitoes was collected at 14 dpi and ZIKV titres were measured using $TCID_{50}$ assay. No ZIKV was detected in the saliva of homozygous TZIKV-C mosquitoes (n=46). Presence of ZIKV in the saliva was detected in 74.4% (29/39) of heterozygous TZIKV-C mosquitoes; however, here again the ZIKV titres were significantly ($p<0.001$) lower (~3 logs) as compared to Higgs WT (Figure 2.2D, Table S2.2).

To determine whether the synthetic small RNAs are broadly inhibitory for ZIKV, vector competence of transgenic TZIKV-C mosquitoes was also assessed using a second contemporary ZIKV strain (PRVABC59, isolated from US traveller to Puerto Rico in 2015, GenBank KU501215). For this strain, seven out of eight ZIKV-targeting synthetic small RNA target sites (although not the same seven as for the Cambodia strain) are 100% conserved with the French Polynesia strain against which the small RNAs were designed (Figure S2.1). Tests for infection, dissemination, and transmission were carried out as above, and the results were comparable to those obtained with the Cambodia strain. Briefly, at 4 dpi, none of the TZIKV-C mosquitoes homozygous for the transgene (n=32) were positive for ZIKV infection in their midguts, and while ZIKV infection was detected in 81.25% (26/32) of the TZIKV-C mosquitoes that were heterozygous for the transgene, these had significantly ($p<0.001$) lower viral RNA levels (~2 logs) than Higgs WT (Figure 2.2E, Table S2.2). TZIKV-C mosquito carcasses and dissected midguts at 14 dpi showed that none of the homozygous TZIKV-C mosquitoes (n=70) were positive for viral replication in either the midgut or the carcass by real-time RT-qPCR, while 70% (49/70) of heterozygous mosquitoes had ZIKV in both the carcass and midgut, albeit with significantly

($p<0.001$) lower levels of viral RNA (~3 logs) than Higgs WT (Figure 2.2F-G, Table S2.2). Finally, ZIKV titre measurements on saliva from individual mosquitoes at 14 dpi demonstrated that no ZIKV was present in homozygous TZIKV-C mosquitoes (n=70), indicating that they would be unable to transmit the virus. Prevalence of ZIKV in saliva was detected in 70% (49/70) of TZIKV-C heterozygous mosquitoes; however, here again the ZIKV titres were significantly ($p<0.001$) lower (~3 logs) as compared with Higgs WT (Figure 2.2H, Table S2.2).

2.4.4 Engineered mosquitoes outperform *Wolbachia*

We next compared the inhibitory effect of our synthetic small RNAs to ZIKV inhibition previously shown with *Wolbachia* (Caragata, Dutra, and Moreira 2016; Schultz et al. 2017; Dutra et al. 2016; Aliota et al. 2016). Vector competence results revealed that midguts from mosquitoes (Higgs WT strain) infected with *Wolbachia* (*w*Mel strain; n=50) had significantly ($p<0.001$) reduced ZIKV (Puerto Rican strain) RNA levels (~2 logs) at 4 dpi compared with uninfected Higgs WT (n=32; Figure 2.2E, Table S2.2). Similarly, viral dissemination at 14 dpi was also reduced ($p<0.001$) in *w*Mel mosquitoes (~3 logs, n=50; Figure 2.2F-G, Table S2.2), and ZIKV titres in mosquito saliva at 14 dpi were significantly ($p<0.01$) lower (~2 logs) in *w*Mel mosquitoes than in uninfected Higgs WT (Figure 2.2H, Table S2.2). Importantly, comparison to the TZIKV-C mosquitoes revealed that the TZIKV-C mosquitoes are significantly ($p<0.001$) more effective as homozygotes, and modestly more effective as heterozygotes ($p<0.05$), at blocking ZIKV infection compared with *Wolbachia*-infected mosquitoes.

2.4.5 Anti-ZIKV transgene inhibits ZIKV transmission in a mouse model

To further characterize ZIKV inhibition by the anti-ZIKV transgene, we also conducted limited tests of *in vivo* transmission capacity on heterozygous TZIKV-C mosquitoes. Specifically, we utilized a very sensitive STAT knockout (*Stat1*-/-) mouse model, in which challenge with ZIKV (either intraperitoneally or via feeding by infected mosquito) rapidly causes systemic infections presenting high virema, resulting in significant weight loss, brain infections, and mortality (Kuo et al. 2018). Firstly, we infected adult female mosquitoes (Higgs WT and TZIKV-C) with Puerto Rican ZIKV strain (PRVABC59) via thoracic injection, which bypasses the midgut barrier resulting in a significant viral titre in mosquitoes (Kuo et al. 2018). At 7 dpi, TZIKV-C (n=28) and Higgs WT (n=29) mosquitoes were separately pooled into four groups (with 6-12 individuals per group; Figure 2.3A) and each group was allowed to blood feed on a *Stat1*-/- mouse, after which mouse weight and survival were measured daily. All mice fed on by infected Higgs WT mosquitoes (n=4) became viremic and died prior to 8 dpi with significant weight loss prior to death ($p<0.05$; Figure 2.3B, C). Conversely, out of the four mice fed on by TZIKV-C mosquitoes, only one showed mortality (albeit at a later date - 12 dpi), and no significant weight loss was observed compared to the control group ($p<0.0001$; Figure 2.3B, C). Measurement of ZIKV titres in the individual mosquitoes utilized for this assay demonstrated that nearly all TZIKV-C mosquitoes had significantly reduced viral titers (~2 log) at 7 dpi compared to Higgs WT ($p<0.0001$; Figure 2.3A).

To better simulate how mosquitoes naturally obtain pathogens (i.e., from blood feeding), we also performed the above assay with mosquitoes that were infected with ZIKV (strain PRVABC59) via oral membrane blood feeding, and obtained similar results. Specifically, at 14 dpi via oral membrane blood feeding, TZIKV-C (n=16) and Higgs WT (n=20) mosquitoes were

pooled into groups of 6-10 and allowed to feed on, and transmit the virus to, *Stat1*-/- mice (n=2 for each transgenic and Higgs WT; Figure 2.3D). The mice fed on by infected Higgs WT mosquitoes experienced significant weight loss and mortality prior to 8 dpi ($p<0.0001$; Figure 2.3E, F). Conversely, mice fed on by TZIKV-C mosquitoes showed no significant change in weight and no infection-associated mortality ($p<0.0001$, Figure 2.3E, F). Viral titre assays on these mosquitoes (at 14 dpi) indicated that ZIKV infection rate was dramatically reduced in TZIKV-C mosquitoes (39% of mosquitoes infected compared to 93% of Higgs WT; Figure 2.3D), and that viral titres of the TZIKV-C mosquitoes that were infected were significantly lower (~2 log) than that of Higgs WT ($p<0.0001$, Figure 2.3D). Altogether, these results demonstrate that the anti-ZIKV transgene confers robust refractoriness to ZIKV infection and transmission, and that even mosquitoes heterozygous for the transgene are unlikely to be able to transmit the virus.

2.4.6 Impact of anti-ZIKV transgene on mosquito fitness

Finally, to determine whether the anti-ZIKV transgene had any significant fitness effects on transgenic mosquitoes, we assessed several fitness parameters including larval to pupal development time, male and female fecundity and fertility, male mating success, adult wing length (as a proxy for body size), and longevity (Table S2.3). No significant differences between Higgs WT and TZIKV-C mosquitoes were observed when examining male mating success, fecundity, and fertility (all p values > 0.9); female fecundity ($p>0.05$); and male and female wing length ($p>0.05$). Conversely, we observed a significant difference ($p<0.01$) in hatching rates of eggs laid by Higgs WT versus TZIKV-C females (with the latter having lower hatching rates), and a significant difference between larval to pupal development time ($p<0.01$), with TZIKV-C individuals developing faster. When assessing adult mosquito survivorship, no significant

differences were observed between Higgs WT and TZIKV-C males (p>0.05; Table S2.3, Figure S2.5), while Higgs WT females survived slightly longer than TZIKV-C females (p<0.0001; Table S2.3, Figure S2.5). Furthermore, there was no significant difference in survival at 14 dpi between Higgs WT and TZIKV-C mosquitoes infected with the Cambodia ZIKV strain (p>0.05; Table S2.4), and similarly no significant difference in survival between Higgs WT, Higgs *w*Mel infected, and TZIKV-C mosquitoes infected with the Puerto Rico ZIKV strain (p>0.05; Table S2.4). Based on the above observations, it appears that although the anti-ZIKV transgene did negatively affect female mosquito longevity and egg hatching rate, it did not result in significant changes to most fitness parameters measured, including larval to pupal development time, fecundity and fertility, male mating success, and body size.

2.5 Discussion

Taken together, our results demonstrate that targeting conserved genes in the ZIKV genome by expressing an engineered polycistronic cluster of synthetic small RNAs confers homozygous mosquitoes complete refractoriness to multiple strains of ZIKV infection, dissemination, and transmission, presumably because they harbor two copies of the anti-ZIKV transgene. Although incomplete, heterozygous mosquitoes also display partial refractoriness to ZIKV infection, dissemination, and transmission, with significant reduction of viral titres in the saliva (>2 logs compared to WT). This significant reduction of ZIKV is greater than the viral inhibition effect of *Wolbachia*, and may be enough to ensure these heterozygous mosquitoes are unable to transmit ZIKV to a susceptible host in the wild. Indeed, this latter point is supported by our finding that heterozygotes were largely unable to transmit ZIKV to immunocompromised (*Stat1* -/-) mice after infection via thoracic injection, and completely unable to transmit ZIKV after

infection via membrane feeding. As intrathoracic injection generates unnaturally high infection levels by bypassing the mosquito midgut and lumen barriers (Kuo et al. 2018), it is perhaps unsurprising that one mouse (possibly fed on by one of the mosquitoes with relatively high viral titres; Figure 2.3A) experienced ZIKV-associated mortality. However, the observation that most of the heterozygous anti-ZIKV mosquitoes infected via thoracic injection - and all of the mosquitoes infected via membrane feeding - were unable to transmit ZIKV to a susceptible mouse model strongly suggests that even heterozygotes are unlikely to be capable of ZIKV transmission in the wild.

While the robust resistance observed in thoracic injection experiments may seem unexpected because this method of infection bypasses the midgut where the CP promoter is canonically expected to drive expression, our RT-PCR data indicated that the anti-ZIKV small synthetic RNAs were expressed in tissues besides the midgut. This is likely due to genomic position effects, which have been previously observed with this promoter (A. W. E. Franz et al. 2011), and could be addressed in future work by optimization of midgut-specific expression (although RNA sequencing data indicates that CP is actually expressed in multiple tissues besides the midgut across various developmental time points (Akbari et al. 2013; Benjamin J. Matthews et al. 2017), and therefore the CP promoter may also act systemically).

Previously in *Ae. aegypti*, resistance to DENV has been engineered by transgenic activation of antiviral pathways (Jupatanakul et al. 2017), transgene-based RNAi in either the midgut (A. W. Franz et al. 2006; A. W. E. Franz et al. 2014) or salivary glands (Mathur et al. 2010), and antiviral hammerhead enzymes (Mishra et al. 2016), and expression of synthetic miRNAs has also been demonstrated to induce partial resistance to DENV-3 and CHIKV(Yen et al. 2018). However, similar approaches have not been successfully demonstrated for ZIKV; and, the currently

described system is potentially especially advantageous, since targeting 6/10 conserved protein-coding genes from the ZIKV genome with 8 separate synthetic small RNAs may reduce the possibility of escapee mutants and thus ensure evolutionary stability.

That said, it remains uncertain how many synthetic small RNAs are necessary to ensure robust disease refractoriness and evolutionary stability in a wild population. In our small RNA sequencing efforts we only detected expression/processing of 5 out of the 8 synthetic small RNAs, suggesting that perhaps the small RNA processing machinery is overloaded, that the CP promoter is not strong enough to ensure robust expression from all 8 synthetic small RNAs, or possibly that some synthetic small RNAs are unstable and get quickly degraded after processing. The latter hypothesis is supported by the fact that expression was only detected from synthetic small RNAs 1, 2, 4, 6, and 8, while 3, 5, and 7 were undetected; given that these are arranged numerically in a linear array (i.e., 1-8), small RNAs 3, 5, and 7 must have been expressed/processed in order for 4, 6, and 8 to be expressed/processed/detected. Moreover, contrary to expectation when using a blood meal inducible promoter such as CP, levels of multiple synthetic small RNAs were lower in post blood meal transgenic samples than in non-blood fed ones. The lack of clear blood meal expression induction is not inconsistent with previous findings regarding use of the CP promoter to drive transgene expression (A. W. E. Franz et al. 2011), and likely arises due to genomic position effects associated with transgene integration site. In any case, even without full expression driven by the CP promoter, it is clear that synthetic small RNA levels are sufficient enough to bring about robust ZIKV resistance in multiple genetic backgrounds. However, future efforts should be focused on addressing the above-mentioned open questions regarding small RNA processing and specificity of expression.

Altogether, this strategy may provide a suitable "cargo" gene for practical use with a gene drive system to reduce/eliminate vector competence of mosquito populations. For example, previous reports have shown that Cas9-mediated homing based gene drive can be used for population modification of the malaria vector mosquito, *Anopheles stephensi* (Gantz et al. 2015), and it should be possible to develop similar systems in *Ae. aegypti*. Given that homing based drive systems quickly convert heterozygotes into homozygotes (Champer, Buchman, and Akbari 2016), linking the anti-ZIKV transgene such as the one described here to such a system could quickly convert an entire mosquito population into anti-ZIKV homozygotes that would be 100% resistant to ZIKV transmission. Recent ZIKV outbreaks have shown that vector control remains an essential part of reducing the health burden of emerging arboviruses. Although the aim of this study was to illustrate feasibility of producing ZIKV-refractory mosquitoes, similar genetic engineering strategies could be used to develop (or improve on (Yen et al. 2018)) single transgenes that render mosquitoes completely resistant to multiple arboviruses like DENV and CHIKV. Given the increasing incidence of these viral infections worldwide, such transgenes (coupled with gene drive systems) can provide an effective, sustainable, and comprehensive strategy for reducing the impact of arboviral mosquito-borne diseases.

2.6 Acknowledgements

This chapter, in full, is a reprint of the material as it appears in the Proceedings of the National Academy of Sciences of the United States of America (PNAS). Anna Buchman[1], Stephanie Gamez[1], Ming Li, Igor Antoshechkin, Hsing-Han Li, Hsin-Wei Wang, Chun-Hong Chen, Melissa J. Klein, Jean-Bernard Duchemin, Prasad N. Paradkar, and Omar S. Akbari. "Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic miRNAs". PNAS. (2019). [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

2.7 Figures

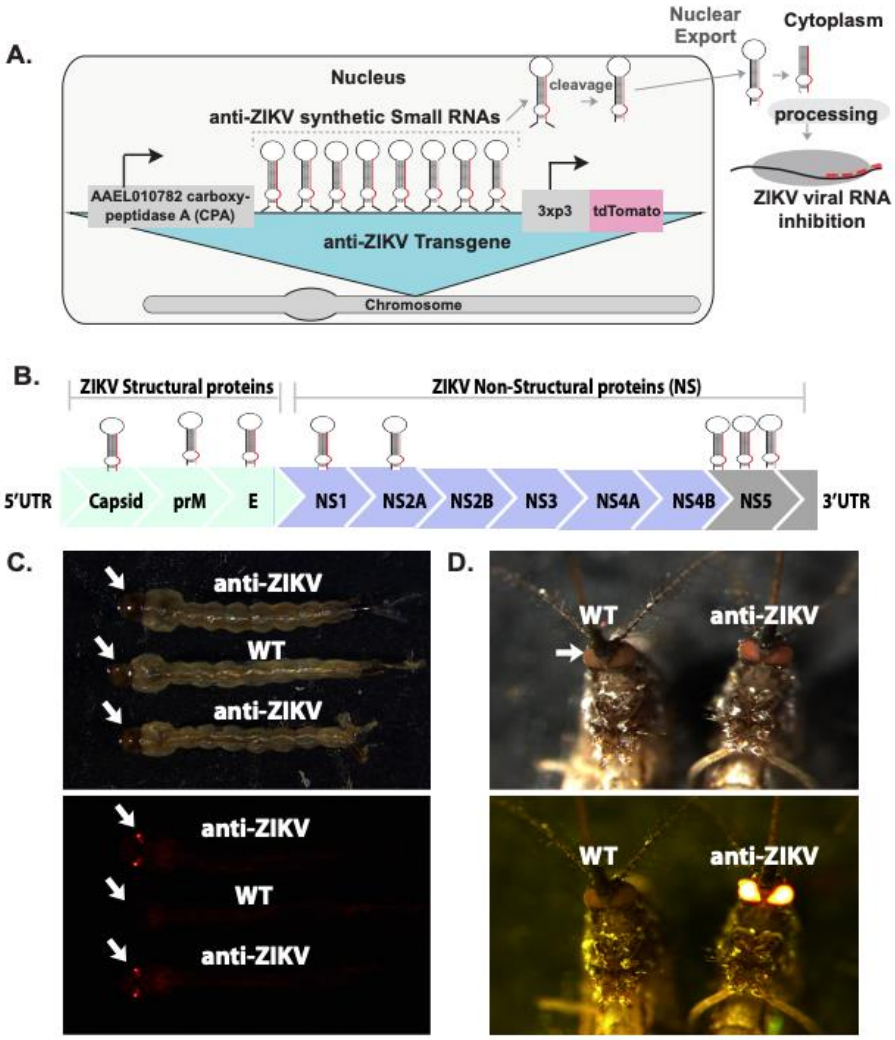


Figure 2.1: Schematic of anti-ZIKV transgene, ZIKV target sites and phenotype of transgenic mosquitoes. A schematic of the anti-ZIKV transgene used in the study consisting of a carboxypeptidase A (AAEL010782; CP) promoter driving expression of a polycistronic cluster of eight synthetic small RNAs engineered to target conserved genes in the ZIKV genome. Following processing, the small RNAs and their target ZIKV viral RNA interact in the cytoplasm (A). A schematic of the ZIKV genome consisting of three structural proteins (Capsid, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) with relative synthetic small RNA targets indicated by hairpin above (B). Higgs WT and TZIKV-C adult mosquitoes (C) imaged under both transmitted light and a fluorescent dsRED filter.

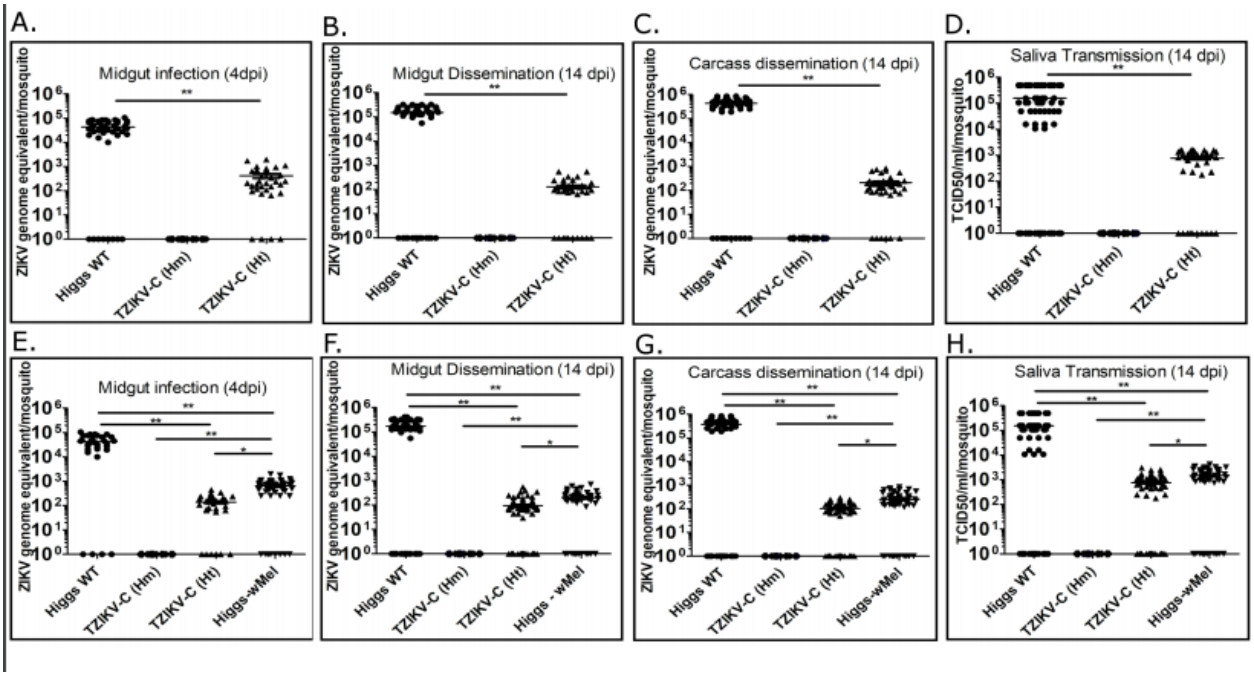


Figure 2.2: ZIKV replication and titres in Higgs WT, TZIKV-C and Wolbachia infected mosquitoes challenged with either a Cambodian or a Puerto Rican ZIKV strain. ZIKV virus genome copies and titres in Higgs wildtype (Higgs WT), TZIKV-C homozygous (Hm) and heterozygous (Ht) transgenic mosquitoes, and Wolbachia-infected Higgs WT (Higgs-wMel) following a blood meal infected with a Cambodian (FSS13025, A-D) or Puerto Rican (PRVABC59, E-H) strain of ZIKV are shown. ZIKV genome equivalent from mosquito midgut (day 4 (A, E) and day 14 (B, F) post infection, dpi) and carcass (14 dpi (C, G)) of Higgs WT and transgenic mosquitoes were determined using real-time RT-qPCR and calculated using previously published methods. Virus titres in the saliva collected from Higgs WT and transgenic mosquitoes 14 dpi were determined using TCID50 on Vero cells and plotted (D, H). Circles represent Higgs WT mosquitoes; diamonds represent TZIKV-C Hm transgenic mosquitoes; triangles represent TZIKV-C Ht mosquitoes; upside down triangles represent Higgs-wMel mosquitoes. Horizontal bars represent the mean virus titre. *represents $p<0.05$ and **represents $p<0.001$. For each experiment, data from three replicates are pooled.

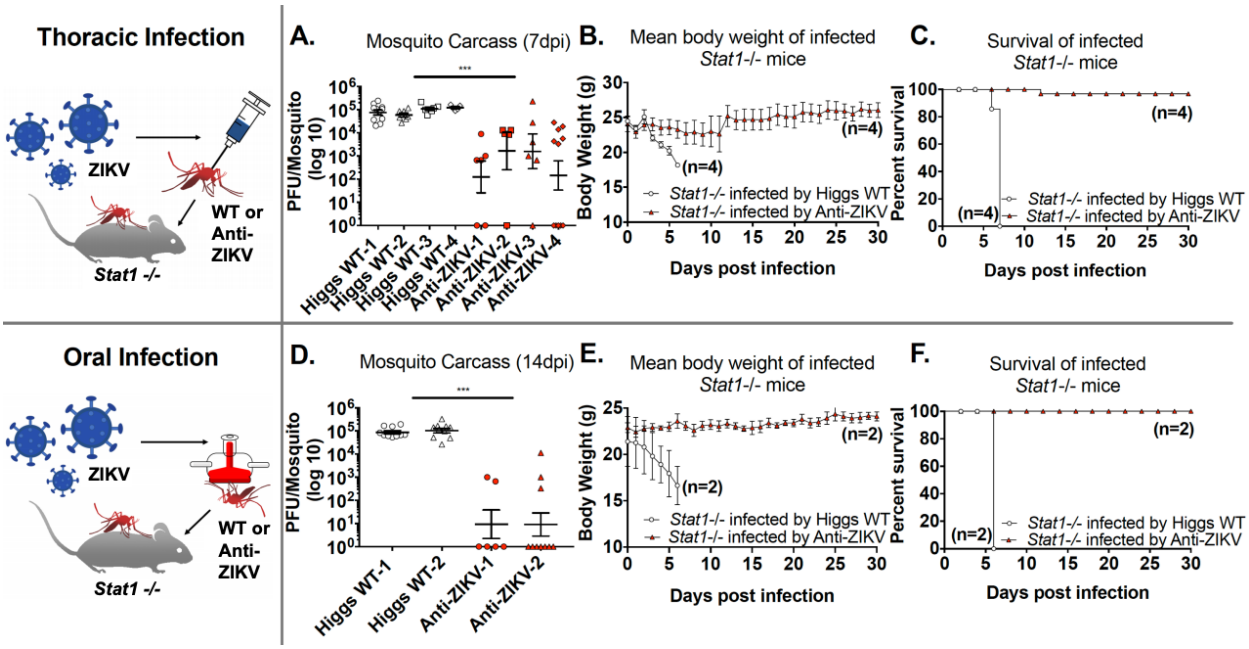


Figure 2.3: Effect of anti-ZIKV transgene on ZIKV transmission in a mouse model. Higgs wildtype (WT) and heterozygous TZIKV-C mosquitoes were infected with ZIKV strain PRVABC59 thoracically (A-C) or orally (D-F), and assayed for their ability to transmit ZIKV to immunocompromised Stat -/- mice. Viral titres in carcasses of mosquitoes infected thoracically (measured at 7 dpi, A) and orally (measured at 14 dpi, D) were determined by plaque assay in Vero cells and plotted. Mean body weight (B, E) and survival (C, F) of Stat -/- mice following ZIKV infection by thoracially (B, C) or orally (E, F) infected Higgs WT and TZIKV-C mosquitoes were measured and plotted. For all plots (A-F), white shapes represent results from Higgs WT mosquitoes; red shapes represent TZIKV-C mosquitoes. For viral titre plots, horizontal bars represent the mean virus titre, and vertical bars represent SEM. For mean body weight plots, vertical bars represent SEM. ***represents $p<0.0001$.

2.8 Supplemental Figures

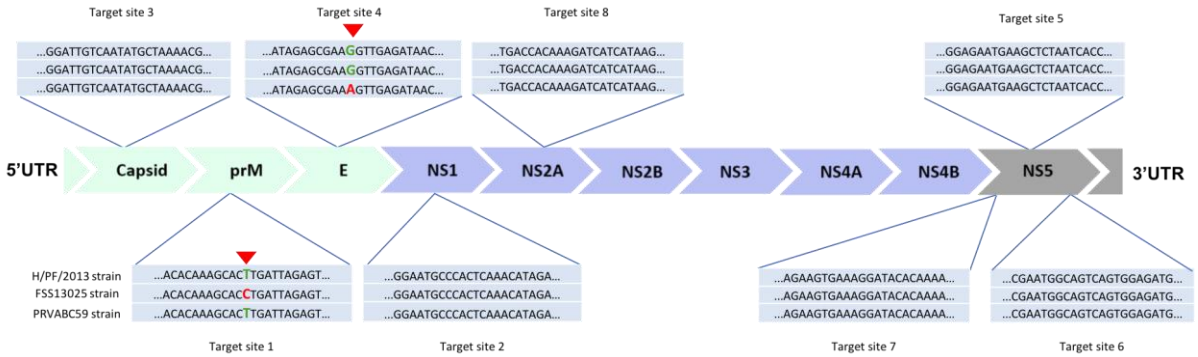


Figure S2.1: Small RNA target site conservation between ZIKV strains H/PF/2013, FSS13025, and PRVABC59. small RNA target sites between the ZIKV strain used for small RNA target selection (H/PF/2013, top sequence) and the strains used for mosquito challenges (FSS13025, middle sequence; PRVABC59, bottom sequence) are highly conserved, with only one base pair mismatch in one target site in each strain (shown in red).

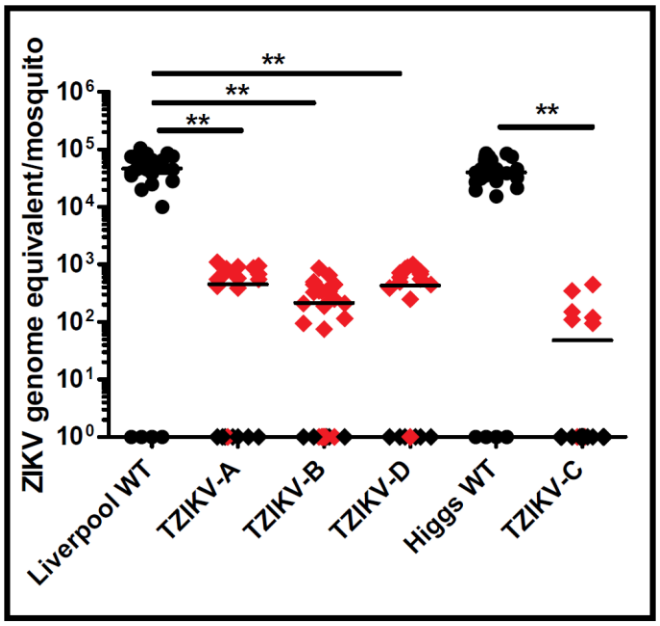
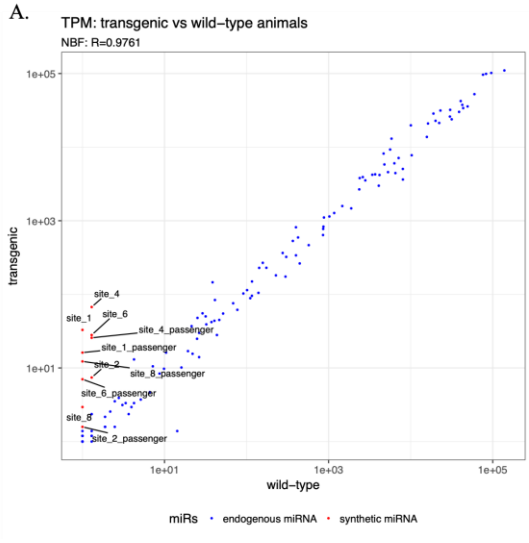


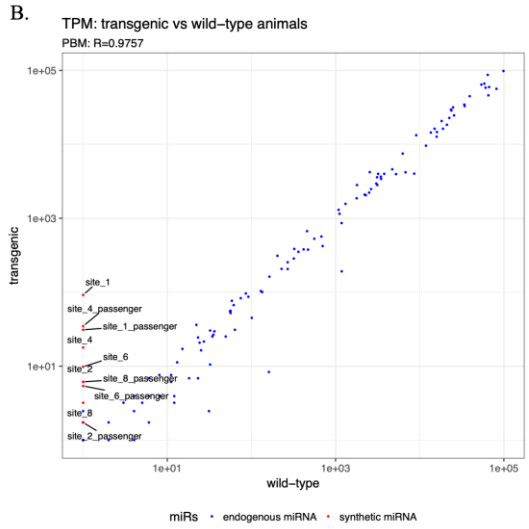
Figure S2.2: Effect of anti-ZIKV transgene on ZIKV titres in four independent mosquito lines. ZIKV virus titres in wildtype (Liverpool WT and Higgs WT), anti-ZIKV transgenic mosquito lines (TZIKV-A, TZIKV-B, TZIKV-C, TZIKV-D) following a blood meal infected with a Cambodian (FSS13025) are shown. ZIKV genome equivalent from mosquito midgut (day 4 post infection) of Liverpool WT, Higgs WT, and transgenic mosquitoes were determined using real-time RT-qPCR and calculated using previously published methods. Circles represent WT mosquitoes; black diamonds represent anti-ZIKV Hm transgenic mosquitoes; red colored diamonds represent anti-ZIKV Ht transgenic mosquitoes. Horizontal bars represent the mean virus titer. Mantel-Cox test was used for statistical analysis. **represents $p<0.001$.

Figure S2.3: Differential expression analysis of small RNAs from Higgs WT and TZIKV-C mosquito midguts. TPM (transcripts per million) values for transgenic versus Higgs WT animals without a blood meal (**A**) and 24 hours after a blood meal (**B**) are shown. Expression of synthetic small RNAs does not affect expression levels of endogenous miRNAs significantly (correlation coefficients of 0.9761 and 0.9757, respectively). MA (log2FoldChange vs. baseMean) (**C**) plot demonstrates that detected synthetic small RNAs are strongly differentially expressed between Higgs WT and transgenic animals.

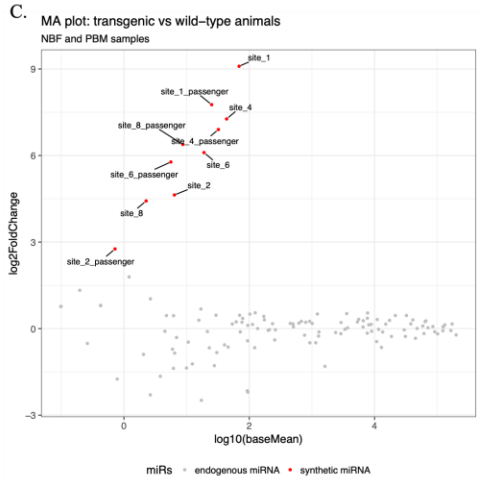
A.


TPM: transgenic vs wild−type animals
NBF: R=0.9761
transgenic
wild−type
1e+01
1e+03
1e+05
site_4
site_1
site_6
site_4_passenger
site_1_passenger
site_2
site_8_passenger
site_6_passenger
site_8
site_2_passenger
miRs
endogenous miRNA
synthetic miRNA

B.


TPM: transgenic vs wild−type animals
PBM: R=0.9757
transgenic
wild−type
1e+01
1e+03
1e+05
site_1
site_4_passenger
site_1_passenger
site_4
site_6
site_2
site_8_passenger
site_6_passenger
site_8
site_2_passenger
miRs
endogenous miRNA
synthetic miRNA

C.


MA plot: transgenic vs wild−type animals
NBF and PBM samples
log2FoldChange
log10(baseMean)
site_1
site_1_passenger
site_4
site_8_passenger
site_4_passenger
site_6
site_6_passenger
site_2
site_8
site_2_passenger
miRs
endogenous miRNA
synthetic miRNA

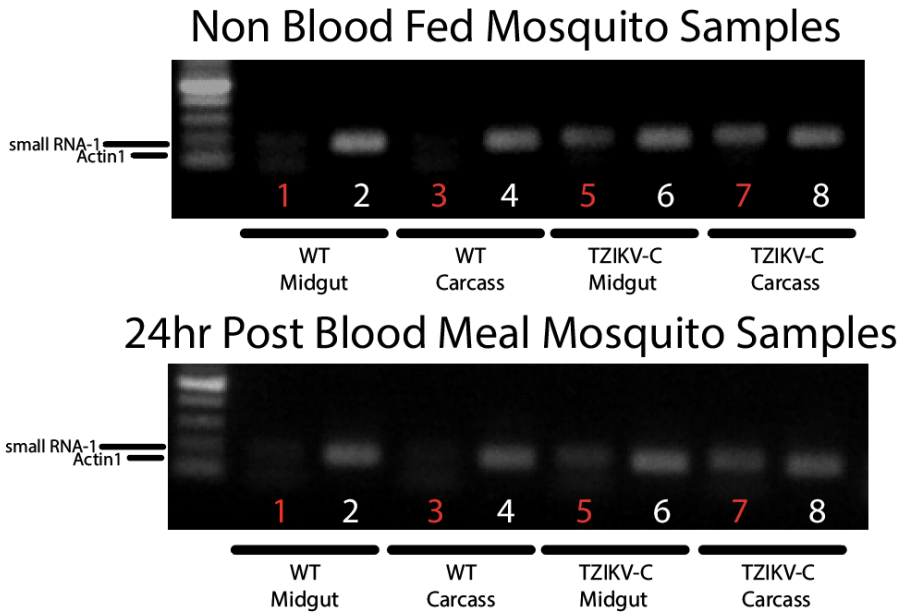


Figure S2.4: RT-PCR analysis on non blood fed and 24-hr post blood fed Higgs WT and TZIKV-C female midgut and carcass samples. A 195bp region of the anti-ZIKV transgene, from the 5'UTR region of the carboxypeptidase A (AAEL010782) promoter to the loop-target site-1 region, was amplified to confirm expression of the anti-ZIKV transgene (odd numbered lanes, labeled in red). A 175bp region of the *Actin1* gene was amplified as a control (even numbered lanes, labeled in white). Higgs WT midgut (lanes 1 and 2), Higgs WT carcass (lanes 3 and 4), TZIKV-C midgut (lanes 5 and 6), and TZIKV-C carcass (lanes 7 and 8) samples were assayed in both a non blood fed (top panel) and 24-hr blood fed (bottom panel) state. All PCR products were sequenced to confirm product identity.

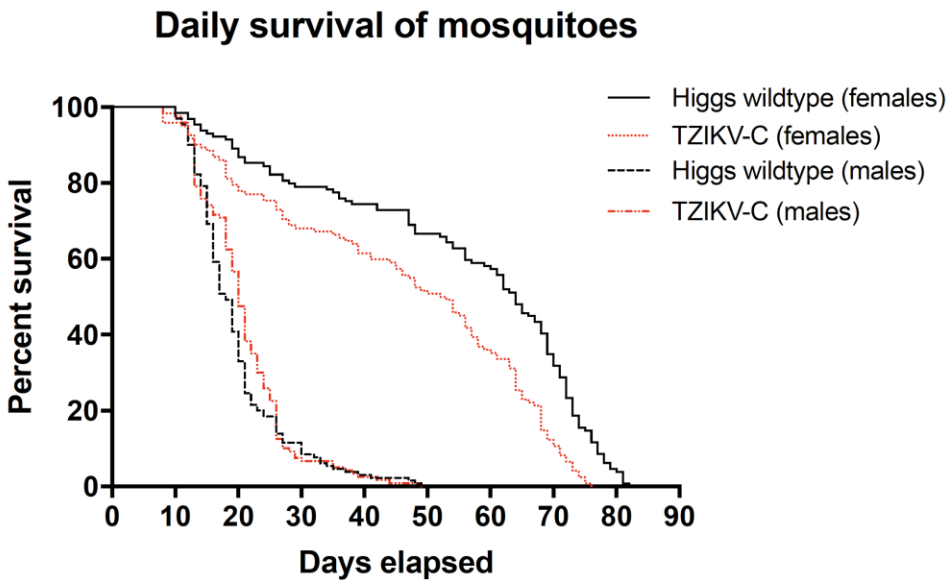


Figure S2.5: Survivorship curve of Higgs WT and TZIKV-C male and female mosquitoes. The x-axis indicates the number of elapsed days after the start of the experiment, and the y-axis indicates the percent of mosquitoes surviving on each elapsed day. Each line represents accumulated results from 120-130 adult mosquitoes combined from 3 biological replicates.

2.9 Supplemental Tables

Table S2.2: Anti-ZIKV transgene effect on ZIKV infection, dissemination, and transmission rates. ZIKV infection rates were quantified in the midgut at 4 days post infection (dpi). Dissemination rates were quantified in both the midgut and carcass at 14 dpi. Transmission rates were calculated by measuring prevalence of ZIKV in the saliva at 14 dpi. For each experiment, data from three replicates is pooled.

Mosquito Line	**Zygosity**	**Viral Strain**	**Infection (Midgut 4 dpi)**	**Dissemination**		**Transmission (Saliva 14 dpi)**
				Midgut (14 dpi)	**Carcass (14 dpi)**	
Higgs WT	N/A	FSS13025	42/50 (84%)	52/65 (80%)	52/65 (80%)	48/65 (73.8%)
TZIKV-C	Heterozygote	FSS13025	28/32 (87.5%)	29/39 (74.4%)	29/39 (74.4%)	29/39 (74.4%)
TZIKV-C	Homozygote	FSS13025	0/32 (0%)	0/46 (0%)	0/46 (0%)	0/46 (0%)
Higgs WT	N/A	PRVABC59	28/32 (87.5%)	53/70 (75.7%)	53/70 (75.7%)	53/70 (75.7%)
TZIKV-C	Heterozygote	PRVABC59	26/32 (81.25%)	49/70 (70%)	49/70 (70%)	49/70 (70%)
TZIKV-C	Homozygote	PRVABC59	0/32 (0%)	0/70 (0%)	0/70 (0%)	0/70 (0%)
Higgs-*w*Mel	N/A	PRVABC59	42/50 (84%)	38/50 (76%)	38/50 (76%)	38/50 (76%)

Table S2.3: Fitness evaluation of Higgs WT and TZIKV-C mosquitoes. Comparisons of several fitness parameters (leftmost column) between Higgs WT (second column from left) and TZIKV-C mosquitoes (third column from left) suggest that there are few significant differences (rightmost column) between the two groups, indicating that the anti-ZIKV transgene does not have a major impact on mosquito fitness.

	Mosquito Strain		
Fitness Parameter	**Higgs WT (N)**	**TZIKV-C (N)**	***P*-value**
Female fecundity[θ†§]	102.5 ± 3.8 (65; 6,648)	113.2 ± 4.4 (65; 7,117)	0.0708
Egg hatchability[θǀ§]	70.9 ± 2.7 (63; 4,684)	58.5 ± 2.8 (63; 4,130)	0.0019
Male mating success[θo¶]	92 ± 0.05 (25)	92 ± 0.05 (25)	>0.9999
Male fecundity[θl§]	118.2 ± 11.8 (25; 2,846)	119.2 ± 12.7 (25; 3,089)	0.9580
Egg hatchability[θIII§]	61 ± 5.8 (23; 1,562)	60.7 ± 4.8 (23; 1,771)	0.9709
Larval to pupal development in days[θ§]	10.35 ± 0.07 (1,224)	9.836 ± 0.10 (904)	0.002
Female wing length[θ§]	3.65 ± 0.02 (56)	3.62 ± 0.08 (58)	0.1489
Male wing length[θ§]	2.76 ± 0.01 (54)	2.79 ± 0.01 (55)	0.0600
Female median survival in days[††]	64 (124)	52 (124)	<0.0001
Male median survival in days[††]	18 (130)	20 (120)	0.2195
% Survival at 14 dpi with ZIKV[θ‡††]	80.6 ± 3.5 (129)	75 ± 4.6 (88)	0.3636

[θ]Mean ± SEM reported.

[†]Average number of eggs laid per female (Number of females scored; total number of eggs counted).

[ǀ]Percentage of laid eggs that produced larvae (Number of females scored; total number of larvae counted).

[o]Percentage of single male outcrosses that gave rise to viable progeny.

[l]Average number of eggs laid per single male outcross (Number of male outcrosses scored; total number of eggs counted).

[III]Percentage of laid eggs that produced larvae per single male outcross (Number of male outcrosses scored; total number of larvae counted).

[§]Unpaired t test with Welch's correction was used.

[¶]Unpaired t test was used to evaluate the statistical significance between the proportions of fertile males.

[††]Mantel-Cox test was used.

[‡]Percentage of infected mosquitoes surviving at 14 dpi.

Table S2.4: The survivorship of ZIKV-infected TZIKV-C mosquitoes at 14 days post infection (dpi). Higgs WT, Higgs *w*Mel+, and TZIKV-C mosquitoes infected with ZIKV strain FSS13025 or PRVABC59 were assessed for survival at 14 dpi. The mean percentage±SEM of surviving mosquitoes and number of mosquitoes tested (in parentheses) are reported. No assay was performed for Higgs *w*Mel mosquitoes infected with strain FSS13025. The Mantel-Cox test was used to compare the survival of infected Higgs WT, Higgs *w*Mel (for PRVABC59 strain only), and TZIKV-C mosquitoes.

	Mosquito strains			
Virus strain	**Higgs WT**	**Higgs WT *w*Mel**	**TZIKV-C**	**P-value**
FSS13025 (Cambodia)	64.1% ± 6.6 (53)	--	73.6% ± 6.1 (53)	0.2528
PRVABC59 (Puerto Rico)	92.1% ± 3.0 (76)	86.9% ± 5.0 (46)	77.1% ± 7.0 (35)	0.0817

2.10 References

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Chapter 3: Broad dengue neutralization in mosquitoes expressing an engineered antibody

3.1 Abstract

With dengue virus (DENV) becoming endemic in tropical and subtropical regions worldwide, there is a pressing global demand for effective strategies to control the mosquitoes that spread this disease. Recent advances in genetic engineering technologies have made it possible to create mosquitoes with reduced vector competence, limiting their ability to acquire and transmit pathogens. Here we describe the development of Aedes aegypti mosquitoes synthetically engineered to impede vector competence to DENV. These mosquitoes express a gene encoding an engineered single-chain variable fragment derived from a broadly neutralizing DENV human monoclonal antibody and have significantly reduced viral infection, dissemination, and transmission rates for all four major antigenically distinct DENV serotypes. Importantly, this is the first engineered approach that targets all DENV serotypes, which is crucial for effective disease suppression. These results provide a compelling route for developing effective genetic-based DENV control strategies, which could be extended to curtail other arboviruses.

3.2 Introduction

Dengue fever is a devastating viral disease caused by several antigenically distinct dengue viral (DENV) serotypes that are all dangerous to humans but cannot be readily controlled using broad-spectrum techniques (Murrell, Wu, and Butler 2011; Mustafa et al. 2015). Transmitted by infected mosquitoes, DENV infection typically manifests as severe fever, headaches, and myalgia (Whitehorn 2001) and can advance to the life-threatening dengue hemorrhagic fever and dengue

shock syndrome (Rajapakse 2011). Global incidences of DENV and its associated economic burden have increased dramatically in recent years (Messina et al. 2014; Bhatt et al. 2013), with over 50% of the world's population now at risk of infection (Brady et al. 2012) and 390 million documented infections per year (Bhatt et al. 2013) for an estimated $40 billion in economic losses annually (Shepard et al. 2016; Selck, Adalja, and Boddie 2014). Moreover, there are currently no specific treatments nor preventive prophylactic measures (World Health Organization 2009) because the single commercially available vaccine (S. R. Hadinegoro et al. 2015) is only partially effective (Capeding et al. 2014), and due to increased risk of severe dengue illness and hospitalization among certain groups, its use is prevented in many contexts ("Dengue Vaccine: WHO Position Paper – July 2016" 2016; Dans et al. 2018; S. R. S. Hadinegoro et al. 2018). Therefore, control of the disease-spreading mosquitoes is currently the best option for preventing DENV transmission ("Dengue Vaccine: WHO Position Paper – July 2016" 2016).

Aedes aegypti (Gibbons and Vaughn 2002), the main vector of DENV and other epidemiologically significant viruses such as chikungunya (CHIKV), yellow fever (YFV), and Zika (ZIKV), is a domestic endophilic mosquito(Scott and Takken 2012) that has expanded its habitable range in recent decades (Gloria-Soria et al. 2014) and will likely continue to spread (Kraemer et al. 2015). Current control measures including the removal of standing water and the use of chemical insecticides have had limited success in reducing *Aedes* populations (Carvalho et al. 2017) and, thereby, incidences of DENV (Haug, Kieny, and Murgue 2016), and can instead cause insecticide resistance and behavioral changes such as a shift in biting times (Gatton et al. 2013; Moyes et al. 2017). Therefore, novel vector control strategies (Yakob et al. 2017), like the use of genetically modified mosquitoes to either suppress mosquito populations or render mosquitoes unable to transmit pathogens (Champer, Buchman, and Akbari 2016), are increasingly

needed. For example, the development and deployment of a genetic Sterile Insect Technique (SIT)-like system termed Release of Insect Dominant Lethal (RIDL) has had some success in reducing *Aedes* mosquito populations in the wild (Aliota et al. 2016; Walker et al. 2011). Moreover, releases of mosquitoes artificially infected with the intracellular endosymbiont *Wolbachia*, which can make infected males incapable of successfully mating with uninfected females in an SIT-like manner and can inhibit mosquito infection with pathogens such as DENV and ZIKV (Aliota et al. 2016; Walker et al. 2011), have also been carried out. These have been intended to either suppress mosquito populations or make them less likely to transmit pathogens, and may hold promise for reducing the incidence of disease (Luciano A. Moreira et al. 2009; Schmidt et al. 2017). However, these technologies require releases of large numbers of insects - and must be carried out on an ongoing basis for RIDL and *Wolbachia*-based SIT - for continued vector control, which is laborious and expensive.

Therefore, there has been increasing interest in the development of engineered gene-drive technologies, which are able to rapidly transmit themselves and any linked "cargo" genes, such as anti-pathogen effectors, through wild disease-transmitting populations (Champer, Buchman, and Akbari 2016; Sinkins and Gould 2006; Macias, Ohm, and Rasgon 2017; Li et al. 2019; Kandul et al. 2019) such that only a few releases of modest amounts of engineered insects could drive desirable cargo genes through wild populations, making them efficient and cost effective for vector control. To achieve disease reduction, such gene-drive systems need to be linked to useful "cargo", such as effective anti-pathogen genes, and several approaches for engineering *Ae. aegypti* resistance to DENV have been attempted. For example, one study used RNA interference by employing inverted RNA repeats to target DENV-2 in a conditional and tissue-specific manner (Franz et al. 2006; Mathur et al. 2010), while another described miRNA cassettes targeting DENV-

3 that reduced viral transmission rates (Yen et al. 2018). In addition to using synthetic small RNAs, others have taken advantage of the mosquito's innate antiviral JAK/STAT pathway to increase resistance to DENV-2 and DENV-4 (Jupatanakul et al. 2017). However, all previous approaches have been limited by their ability to target only one or two—not all four—major DENV serotypes. Because hyperendemicity of DENV in tropical areas is frequent (Messina et al. 2014) and secondary DENV infection has been linked to severe dengue disease (SDD), refractory mosquitoes should be capable of blocking all serotypes or risk being ineffective in controlling dengue epidemics. Therefore, better anti-DENV effectors are needed.

Broadly neutralizing antibodies may be especially promising as anti-DENV effector gene candidates because of their ability to neutralize antigenically diverse viruses (Burton et al. 2012). However, while engineered monoclonal antibodies that confer resistance to *Plasmodium*, a protozoan parasite that causes malaria, have been expressed in *Anophelene* mosquitoes (Isaacs et al. 2012; Sumitani et al. 2013; Isaacs et al. 2011), none targeting a virus have been described in any mosquito species. Previously, a DENV-targeting 1C19 monoclonal antibody (MAb) was identified from a large panel of naturally occurring MAbs from human subjects following vaccination or natural infection (Smith et al. 2013). *In vitro* studies demonstrated that this antibody neutralized viruses from all major DENV serotypes and was capable of significantly reducing viremia in a mouse model after DENV-1 and DENV-2 infection (Smith et al. 2013). Here, we engineer *Ae. aegypti* to express a 1C19-based, broadly neutralizing, single-chain variable fragment (scFv) that is capable of neutralizing all four DENV serotypes (Smith et al. 2013). Crucially, we demonstrate that mosquitoes expressing this anti-DENV scFv cannot be infected with or transmit any of the four DENV serotypes and have few significant fitness costs conferred by the presence

of the antibody. These results provide a promising route for developing effective DENV control strategies using genetically engineered mosquitoes.

3.3 Methods

3.3.1 Anti-DENV scFv design

Sequences for the 1C19 variable heavy and light chains were obtained from hybridoma cells expressing the human monoclonal antibody (Smith et al. 2013) that had been cloned biologically by flow cytometry. RNA was extracted using the RNeasy kit (Qiagen #74104), and RT-PCR amplification of antibody gene cDNAs was performed using primer sets designed to detect all human antibody variable gene sequences (Smith et al. 2013). The sequence of the antibody cDNAs was determined by automated Sanger sequence analysis. The sequence analysis of the antibody variable gene sequences in the cDNAs was performed using the international ImMunoGeneTics information system (IMGT).

The variable regions of 1C19 were joined by a 15-amino-acid repeating glycine-serine [G(4)S]3 linker (Hudson and Kortt 1999) to encode a scFv form of the antibody(Yusakul et al. 2016). These chain regions were codon optimized for *Ae. aegypti* expression and then gene synthesized into a vector (GenScript, Piscataway, NJ). For OA984-HA, a 3’ 30-amino-acid human influenza hemagglutinin (HA) epitope tag with a G(4)S linker (Corby-Harris et al. 2010) was added to the carboxy terminus of the single chain antibody for protein expression verification.

3.3.2 Plasmid assembly

To generate vector OA984 (the anti-DENV scFv-antibody transgene), several components were cloned into the *PiggyBac* plasmid pBac[3xP3-DsRed] (a kind gift from R. Harrell) using

Gibson assembly/enzymatic assembly (EA) cloning (Gibson et al. 2009). First, a *Drosophila* codon-optimized tdTomato marker was amplified with primers 984.1A and 984.1B (Supplementary Table S2 for all primers) from a gene synthesized vector (GenScript, Piscataway, NJ) and cloned into a XhoI/FseI-digested pBac[3xP3-DsRed] backbone using EA cloning. The resulting plasmid was digested with AscI, and the following components were cloned in via EA cloning: the predicted *Ae. aegypti* carboxypeptidase promoter (L. A. Moreira et al. 2000) amplified from *Ae. aegypti* genomic DNA using primers 984.2A and 984.2B, a GFP sequence amplified from vector pMos[3xP3-eGFP] (Kokoza et al. 2001) with primers 984.3A and 984.3B, and a 677-bp p10 3' untranslated region (UTR) amplified with primers 984.4A and 984.4B from vector pJFRC81-10XUAS-IVS-Syn21-GFP-p10 (Addgene plasmid #36432). The anti-DENV scFv was then subcloned into the final vector from a gene-synthesized plasmid (GenScript, Piscataway, NJ) using PmeI and PacI sites and traditional ligation cloning. Annotated plasmid sequences and plasmid DNA are available via Addgene (plasmid #120363).

To generate vector OA984-HA (anti-DENV scFv with HA-epitope tag), the G(4)S linker and HAx3 tag were amplified with primers 984B.C1 and 984B.C2 from the *ninaE*[SBP-His] vector containing these components (Chen, Chen, and Montell 2015) and cloned into the PacI digested OA984 backbone using EA cloning. Annotated plasmid sequences and plasmid DNA are available via Addgene (plasmid #120362). All primer sequences used to generate these plasmids are listed in Table S2.

3.3.3 Generation of transgenic mosquitoes

Germline transformations were carried out largely as described (Li et al. 2017). Briefly, 0–1 hr old Higgs wildtype (WT) *Ae. aegypti* pre-blastoderm embryos were injected with a mixture

of vector OA984 or OA984-HA (200 ng/µL) and a source of *PiggyBac* transposase (200 ng/µL)(Kokoza et al. 2001); the injected embryos were hatched in deoxygenated H_2O. A total of 127 surviving WT adult G_0 males and 115 surviving WT adult G_0 females were recovered after the injection. Microinjected WT G_0 adults were assigned to 48 pools and outcrossed to WT of the opposite sex in medium-sized cages. Larvae were fed ground fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany) and adults were fed with 0.3 M aqueous sucrose. Adult females were blood fed three to five days after eclosion using anesthetized mice. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as recommended by the National Institutes of Health, and the methods were supervised by the local Institutional Animal Care and Use Committee (IACUC). A total of 38,177 WT G_1s were screened. G_1 larvae with strong positive fluorescent signals (3xp3-tdTomato) were selected under the fluorescent stereomicroscope (Leica M165FC) and were separated into six individual groups characterized by fluorescence patterning and intensity. One single transgenic male from each group was then allowed to separately mate with 10 WT females to isolate each independent line. Three independent lines, TADV-A (vector OA984), TADV-B (vector OA984-HA), and TADV-C (vector OA984-HA) with the strongest fluorescence expression patterns were selected for further characterization. To determine whether these lines represented single chromosomal insertions, we backcrossed single individuals from each of the lines for four generations to WT stock and measured the Mendelian transmission ratios in each generation; in all cases, we observed a 50% transmission ratio, indicating single-chromosome insertion. For one of the three lines (TADV-A), transgenic mosquitoes were inbred for at least 20 generations to generate a homozygous stock. Mosquito husbandry was performed under standard conditions as previously described (Akbari et al. 2013).

3.3.4 Characterization of insertion sites

To characterize the insertion site of vector OA984 or OA984-HA in transgenic mosquitoes, we adapted a previously described inverse polymerase chain reaction (iPCR) protocol (Huang, Rehm, and Rubin 2009) as follows. First, genomic DNA (gDNA) was extracted from 10 transgenic fourth instar larvae using the DNeasy Blood & Tissue Kit (Qiagen #69504) per the manufacturer's protocol. Two separate restriction digests were performed on the gDNA (at 100 ng/µL) to characterize the 5' and 3' ends of the *PiggyBac* insertion using Sau3AI (5' reaction) or HinP1I (3' reaction) restriction enzymes. A ligation step using NEB T4 DNA Ligase (NEB #M0202S) was performed on the restriction digest products to circularize digested gDNA fragments, and two subsequent rounds of PCR were carried out per ligation using the corresponding *PiggyBac* primers listed in Table S3. The final PCR products were cleaned up using the MinElute PCR Purification Kit (Qiagen #28004) in accordance with the manufacturer's protocol and were sequenced via Sanger sequencing (Source BioScience, Nottingham, UK). To confirm the transgene insertion locus and orientation via PCR, primers were designed based on iPCR-mapped genomic regions and used in tandem with *PiggyBac* primers based on their location as listed in Table S3. Sequencing data then was blasted to the AaegL5.0 reference genome (Benjamin J. Matthews et al. 2017). The sequencing data was aligned with SeqManPro (DNASTAR, Madison, WI) to determine the orientation of the transgene insertion site. Analysis of the sequencing data indicated that the insertion site for TADV-A is on chromosome 2 (approximate position 310,340,476), the insertion site for TADV-B is on chromosome 2 (approximate position 301,489,980), and the insertion site for TADV-C is on chromosome 1 (approximate position 30,451,048) when aligned to the AaegL5 assembly (GenBank assembly accession: GCA_002204515.1)(B. J. Matthews n.d.).

3.3.5 Total RNA extraction, isolation, and sequencing

Total RNA was extracted from the midguts of non-blood-fed and 24-hours post-blood-fed TADV-A, TADV-B, TADV-C or WT adult females using the Ambion mirVana mRNA Isolation Kit (ThermoFisher Scientific #AM1560). Following extraction, the RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific #AM2238). The RNA quality was assessed using an RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513) and a NanoDrop 1000 UV-vis spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE). mRNA was isolated using an NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490), and libraries were constructed using an NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770). The libraries were quantified using a Qubit dsDNA HS Kit (ThermoFisher Scientific #Q32854) and a High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies #5067-4626) and sequenced on an Illumina HiSeq2500 in single-read mode with a read length of 50 nt and sequencing depth of 30 million reads per library following the manufacturer's instructions. Reads were mapped to the *Ae. aegypti* genome (AaegL5.0) supplemented with the 1C19 cDNA sequence using STAR aligner (Dobin et al. 2013), and the expression levels were determined with featureCounts (Bhadauria 2017) (S4 Table). Correlation coefficients of the transcripts-per-million (TPM) values between WT and transgenic animals were calculated in R[14] and plotted with ggplot2 (Figure S3.1). Differential expression analysis between transgenic and WT sample pairs of the same feeding status (NBF or PBM) for each line using DESeq2 (Love, Huber, and Anders 2014) identified no significantly changed genes (padj < 0.05) for all six comparisons (data not shown). To increase the sensitivity of the assay, two factor analysis using both NBF and PBM samples per line with design = ~ feeding + genotype was also performed and identified a number

of differentially expressed genes for each line (S5 Table). However, expression of only nine genes was consistently altered in all three lines (S6 Table), suggesting that expression of the 1C19 scFv transgene had minimal impact on overall expression patterns of endogenous genes. All sequencing data can be accessed at NCBI SRA (study accession ID PRJNA524725).

3.3.6 Western blot assays

The general western blot protocol was adapted from CSH Protocols: SDS-PAGE of Proteins (Simpson 2006). Briefly, 5–7 days post eclosion, midguts from 25 non-blood-fed and 16-hour post-blood-meal heterozygous TADV-A transgenic and WT mosquitoes were dissected and collected in 1x PBS. Protein samples from dissected tissues were extracted with ice-cold radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP-40, 1 mM EDTA). The protein concentration was measured using Protein Assay Dye (Bio-Rad, Cat. No#5000006) and multi-detection microplate readers (Molecular Devices, SpectraMax M2). Next, 40 µg of total protein were run on a 12% SDS-PAGE and transferred onto a 0.45 µm Immobilon®-P Transfer Membrane (Merck Millipore, Cat. NO#IPVH00010). The membrane was hybridized with a custom antibody at a 1 µg/mL dilution (GenScript, Item number: U3233DA170_2) to directly recognize the 1c19 scFv peptide (26.3KDa) as well as a monoclonal antibody specific to the HA tag for lines TADV-B and C (Cell Signaling, #3724S) at a 1:1,000 dilution; these were subsequently detected by using rabbit IgG antibody (HRP) (GeneTex, Cat. No#GTX 213110-01) at a 1:10,000 dilution. Images were generated by applying the chemiluminescent HRP substrate (Millipore, Cat. No#WBKLS0500) to the blots.

3.3.7 DENV infection of mosquitoes and virus determination

All experiments were performed under biosafety level 3 (BSL-3) conditions in the insectary at the Australian Animal Health Laboratory. The following DENV strains were used for all viral challenge experiments: DENV-1 (isolate ET243, GenBank EF440432), DENV-2 (isolate ET300, GenBank EF440433), DENV-3 (isolate ET209, GenBank EF440434), DENV-4 (isolate ET288, GenBank EF440435). The virus was passaged in Vero cell monolayer cultures before use for mosquito infections. WT or transgenic (confirmed by red fluorescence in the eye) mosquitoes were exposed to DENV as described previously (Duchemin et al. 2017). Briefly, female mosquitoes were challenged with an infected blood meal ($TCID_{50}$ /mL) through membrane feeding using chicken blood and skin. For infection frequency and virus titer, mosquito midguts were collected at 4 dpi. For dissemination and transmission frequency, mosquito saliva, midguts, and carcasses were collected at 14 dpi. Mosquito saliva was used to determine viral titers using a $TCID_{50}$ assay on Vero cell monolayer cultures. Midguts and carcasses were used to determine the presence of viral RNA using RT-qPCR against NS5. Mosquito viral challenge, processing, saliva testing, and molecular analyses of infection and dissemination were carried out as previously described (Duchemin et al. 2017). DENV infection frequency was defined by the number of midguts (day 4) found positive for viral nucleic acid. Similarly, the dissemination frequency was calculated by the number of carcasses (day 14) found positive by qPCR. Transmission frequency was defined by the number of $TCID_{50}$-positive saliva samples over the number tested. These different frequencies and average $TCID_{50}$ values were compared by the Student's two-tailed t-test.

3.3.8 Confirmation of transgene zygosity

Both homozygous and heterozygous (generated by crossing out homozygous individuals to WT) mosquitoes were used for assays. To confirm the zygosity of tested transgenic mosquitoes, mosquito heads were homogenized using a bead-beater device for DNA extraction in 30 μL of extraction buffer (1x Tris-EDTA, 0.1 M EDTA, 1 M NaCl, and 2.5 μM proteinase K) and incubated at 56°C for 5 minutes and then at 98°C for 5 minutes. The first round of PCR was performed to test for the presence of the anti-DENV transgene using primers 991.3F1 and 1018.S19 (S3 Table). Another round of PCR was then performed using primers 1018.S19 and 1018.S21 (S3 Table) to amplify the WT insertion locus (i.e., locus lacking transgene insertion) and thus determine zygosity. For TADV-B, primer set 991.3R2 and 1018.S73 was used to amplify the anti-DENV transgene and primer set 1018.S73 and 1018.S74 were used to amplify the WT insertion site. For TADV-C, primer sets 991.3F2 and 1018.S80 and set 1018.S80 and 1018.S82 were used to amplify the anti-DENV transgene and WT insertion site, respectively. WT mosquitoes served as controls to ensure that the WT locus was successfully amplified in the genetic background. A PCR kit (ThermoFisher Scientific #F553S) with a 57°C annealing temperature was used for all PCRs following standard protocols.

3.3.9 Generation of *w*Mel *Wolbachia* line and infection assay

Eggs of *Ae. aegypti* infected with the *Wolbachia* strain *w*Mel were obtained from the World Mosquito Program (Prof. Scott O'Neill, Monash University). WT mosquitoes infected with *w*Mel were generated by crossing *w*Mel+ females with males from the WT line, and the resulting offspring were used for DENV infection experiments. At the end of the experiment, the *Wolbachia* infection status of these mosquitoes was tested using PCR with primers specific for *w*Mel detection

(Joubert et al. 2016) (S3 Table). The PCRs indicated the presence of *w*Mel in >90% of mosquitoes, and only results from these positive mosquitoes were used for further analysis.

3.3.10 Fitness evaluation on transgenic anti-DENV mosquitoes

To determine if the anti-DENV transgene conferred a fitness cost, several fitness parameters were evaluated in TADV-A transgenic heterozygous and sibling WT mosquitoes. The evaluations of all experimental and control replicates were performed simultaneously. Insectary conditions were maintained at 28°C and 70 to 80% in relative humidity with a 12 hr light/dark cycle. To assess the larval to pupal development time, the eggs were vacuum hatched, and the larvae were distributed into pans (50 larvae per pan) containing 2.5 L of ddH_2O and 0.6 mL of fish food slurry. To determine the larval to pupal development time of transgenic and WT control mosquitoes, the larvae were allowed to pupate, and pupae were collected and counted every day until no pupae were left. To assess female fertility and fecundity, 90 WT and transgenic females were mated to 20 WT males in a cage. After four days, the females were blood fed and individually transferred into plastic vials filled with water and lined with egg paper. After three days, egg papers were collected, and the eggs were counted and vacuum hatched in nine-ounce plastic cups. Starting on the fourth day, the larvae were counted every day until no larvae were present. Female fecundity refers to the number of eggs laid per female, and fertility reflects the number of eggs hatching to produce larvae. To measure male mating success, fecundity, and fertility, one TADV-A transgenic or WT male was mated to five WT females in a single mesh-covered cup filled with water and lined with egg paper. Three days post blood meal, the cups were checked for the presence of eggs, which were collected, counted, and hatched. Hatched larvae were then counted every day until no larvae were present. Male mating success was calculated as the percentage of single male

outcrosses that produced larvae. Fecundity was measured as the number of eggs laid per cup; fertility was determined by the number of hatching larvae in each cup. Finally, to assess mosquito longevity, equal numbers of male and female TADV-A transgenic or WT mosquitoes were placed in medium-sized cages (in triplicate). Mosquitoes that died were counted and removed daily until all mosquitoes had died. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, California, USA). The means were compared using unpaired t tests with Welch's correction, with the exception of male mating success that did not use Welch's correction. The analyses of mosquito survivorship used the Mantel-Cox test. *P* values > 0.05 were considered not significant.

3.4 Results

3.4.1 Generation of DENV-resistant mosquitoes

To determine whether expressing an anti-DENV antibody in mosquitoes could confer resistance to DENV, we first needed to engineer a broadly neutralizing antibody that was compatible with mosquitoes and could be expressed *in vivo* in its desired form. We chose 1C19 as our model due to its ability to cross-neutralize multiple DENV serotypes in humans(Smith et al. 2013). As it is a human monoclonal antibody, however, it cannot be unobtrusively expressed in mosquitoes, so a new form that is both compatible with mosquitoes and maintains its neutralization capabilities had to be designed. We then choose to engineer an scFv comprising the linked variable heavy (VH) and light (VL) chains because this format removes the human-specific constant region that could impart difficulties in a mosquito and it can be expressed in one "chunk" in an organism without the need for additional *in vivo* processing. To do this, sequences for the 1C19 VH and VL

chains were obtained from hybridoma cells expressing the human monoclonal antibody (Smith et al. 2013).

We then engineered a scFv comprising the VH and VL domains of 1C19 linked using a 15-amino-acid repeating glycine-serine [G(4)S]3 linker (Hudson and Kortt 1999) that was codon-optimized for *Ae. aegypti*. We also engineered a version of this 1C19 scFv that was fused with a 3' 30-amino-acid human influenza hemagglutinin (HA) epitope tag, commonly used as a general expression tag, reasoning that it might be useful in downstream expression analyses. To conditionally drive expression of the 1C19 scFvs in the midgut of female mosquitoes following a blood meal, which would ensure 1C19 expression any time the mosquito was in contact with DENV, we used the *Ae. aegypti* carboxypeptidase (CP) promoter (L. A. Moreira et al. 2000), which should induce expression in the midgut following blood ingestion (Figure 3.1A). (Previous findings determined that the CP promoter induces enhanced transcription of *Aedes aegypti* CPA mRNA after a blood meal and a somewhat moderate expression in sugar-fed mosquitoes (Edwards et al. 2000).

The engineered anti-DENV transgenes (termed plasmid OA984 for the untagged version and plasmid OA984-HA for the HA-tagged version) also contained an eye-specific 3xP3 promoter (Berghammer, Klingler, and Wimmer 1999), driving expression of tdTomato as a fluorescent transgenesis marker. Following the typical transgenesis procedure in mosquitoes, consisting of embryonic microinjection and G_0 outcrossing, multiple independent transgenic lines (n = 6) were readily identified in the G_1 generation via the robust expression of tdTomato fluorescence; three of the lines with the strongest marker expression (termed Transgenic Anti-DENV [TADV]-A, containing OA984; and TADV-B and C, containing OA984-HA) were selected for further experiments. We carried out inverse PCR (iPCR) on genomic DNA extracted from the transgenic

mosquitoes to verify the transgene insertion site and performed backcrosses to WT for multiple generations to ensure that the transgenic lines represented single chromosomal insertions, and were able to confirm that, in all three independent lines, the anti-DENV transgenes were stably integrated into single chromosomes.

3.4.2 Expression of 1C19 scFv antibody

Robust expression and processing of 1C19 scFv transcripts is required for proper neutralization of DENV, and it is important to know if such expression perturbs global gene-expression patterns, which might interfere with the fitness of the mosquito. To confirm this, we sequenced total RNA populations from dissected midgut tissues isolated from both blood-fed and non-blood-fed female Higgs wildtype (WT) or TADV-A, B, or C mosquitoes using an Illumina platform. We detected robust expression of the 1C19 scFv mRNA in both non-blood-fed and 24-hour post-blood-fed transgenic mosquitoes of all three lines, with clearly increased expression levels at 24-hours post-blood meal, while no expression was observed in the midguts of female WT mosquitoes, suggesting that expression of the 1C19 scFv antibody is transgene-dependent and blood-meal-inducible (~2.35-14.45 fold) as was intended. Importantly, while there were some changes in expression of some genes in transgenic mosquitoes when compared to WT, these represented a small fraction of the genome and, with a few exceptions, did not appear to be consistent between the three transgenic lines (S5 and S6 Tables). This suggests that the 1C19 scFv expression does not affect gene-expression patterns in a major, global way (Figure S3.1, Tables S3.4-S3.6).

To confirm the proper expression of the 1C19 scFv, we performed western blots on dissected midgut tissue from non-blood-fed and blood-fed WT and TADV-A as well as blood-fed

TADV-B and TADV-C female mosquitoes using either a custom anti-1C19 scFv antibody, or an anti-HA antibody. Blot analyses revealed that the 1C19 scFv peptide was efficiently expressed following a blood meal exclusively in transgenic mosquitoes (Figure S3.2). Altogether, these results suggest that the anti-DENV transgene is stably integrated into the mosquito genome and that the DENV-targeting 1C19 antibody is expressed in an appropriate context (i.e., in the midgut following a blood meal) for DENV suppression.

3.4.3 Engineered mosquitoes are resistant to infection with all four DENV serotypes

To determine the functional significance of anti-DENV 1C19 scFv expression on vector competence, the DENV-2 infection rates of the three transgenic TADV lines were first compared to that of WT. To do this, adult females (WT or TADV-A, B, or C) were exposed to serotype DENV-2 (isolate ET300, Genbank EF440433) via membrane blood feeding. At 4 days post infection (dpi), midguts from blood-fed mosquitoes were dissected, and DENV RNA copies were measured using RT-qPCR. All three lines showed a significant reduction in midgut infection rate (45 to 71%) and viral RNA levels (2 to 3 log lower) compared with WT control mosquitoes (infection rate 92%) (Figure S3.3; Table S3.1). Since no significant difference in DENV-2 midgut infection levels was detected between the three transgenic lines, TADV-A, which exhibited the strongest antiviral phenotype (Figure S3.3; Table S3.1), was selected for further comprehensive characterization.

For a more detailed study of the TADV-A DENV vector competence, the effect of transgene zygosity on the infection rate was determined by exposing additional adult females (WT or TADV-A) to serotype DENV-2 and analyzing the dissected midguts at 4 dpi. Results from three biological replicates revealed that none of the TADV-A mosquitoes homozygous for the transgene

(n = 35) were positive for DENV-2 infection in the midguts (Figure S3.1B). DENV-2 infection was detected in 85.4% (35/41) of the TADV-A mosquitoes that were heterozygous for the transgene; however, these mosquitoes had significantly ($p < 0.001$) lower (~3 $\log_{10}$) viral RNA levels (8.20 x 10^1 genome equivalent [GE]) than the WT (4.25 x 10^4 GE) (Figure 3.1B, Table 3.1).

To assay for viral dissemination to the rest of the mosquito body, total RNA was collected from whole TADV-A mosquito carcasses (without midguts) and dissected midguts from both homozygous and heterozygous mosquitoes at 14 dpi. The results from three biological replicates indicated that none of the homozygous TADV-A mosquitoes (n = 30) were positive for viral replication (dissemination) in either the midgut or the midgut-free carcass (Figure 3.1B, Table 3.1). DENV-2 prevalence was detected in 86.6% (26/30) of heterozygous TADV-A mosquitoes in both the carcass and midgut; however, they also had significantly ($p < 0.001$) lower levels of viral RNA (~3 $\log_{10}$) compared to the WT (Figure 3.1B, Table 3.1). Finally, as transmission occurs through the saliva, viral transmission rates were determined by collecting the saliva from individual mosquitoes at 14 dpi and measuring the DENV-2 titers using an assay for the median tissue culture infective dose ($TCID_{50}$). No DENV-2 was detected in the saliva of homozygous TADV-A mosquitoes (n = 30) (Figure 3.1B), though it was detected in 83.3% (25/30) of heterozygous TADV-A mosquitoes; however, here again the DENV-2 titers were significantly ($p < 0.001$) lower (3.56 x 10^2 $TCID_{50}$/ml/mosquito) than the WT mosquitoes (2.70 x 10^5 $TCID_{50}$/ml/mosquito) (Figure 3.1B, Table 3.1).

To determine whether the anti-DENV 1C19 scFv is broadly inhibitory for other DENV serotypes, the vector competence of TADV-A mosquitoes was assessed using DENV-1 (isolate ET243, GenBank EF440432), DENV-3 (isolate ET209, Genbank EF440434), and DENV-4 (isolate ET288, Genbank EF440435). Tests for infection, dissemination, and transmission were

carried out as above, and the results, presented together in Figure 3.1B and Table 3.1, were comparable to those obtained with the DENV-2 serotype. In short, the TADV-A mosquitoes homozygous for the transgene proved to be refractory to infection with all three additional serotypes also showing no infection in their midguts at 4 dpi (DENV-1 n = 28; DENV-3 n = 30; DENV-4 n = 27). Even at 14 dpi, there was no sign of viral replication in the midgut or carcass for all tested specimens, and none of the saliva samples (DENV-1 n = 28; DENV-3 n = 30; DENV-4 n = 28) were positive for the virus. As with DENV-2, the mosquitoes heterozygous for the transgene still tested positive for the virus in most specimens, though the overall DENV titers were significantly lower than compared to the WT in all cases (Figure 3.1B; Table 3.1).

3.4.4 Engineered anti-DENV mosquitoes outperform *Wolbachia*

To compare the inhibitory effect of the anti-DENV 1C19 scFv to DENV inhibition through *Wolbachia* (O'Neill 2018; Ye et al. 2015; Carrington et al. 2018) infection, we challenged WT mosquitoes infected with *Wolbachia* (*w*Mel) with DENV-2. Vector competence results revealed that midguts from mosquitoes infected with *Wolbachia* had significantly ($p < 0.001$) reduced DENV-2 RNA levels (4.75×10^1 GE) at 4 dpi compared with the WT (4.25×10^4 GE) (Figure 3.1B, Table 3.1). Similarly, viral dissemination at 14 dpi was also reduced ($p < 0.001$) in *w*Mel mosquitoes (~3 $\log_{10}$), and DENV titers in mosquito saliva at 14 dpi were significantly ($p < 0.01$) lower (~3 $\log_{10}$) in *w*Mel mosquitoes (4.90×10^1 $TCID_{50}$/ml/mosquito) than in the WT (2.70×10^5 $TCID_{50}$/ml/mosquito) (Figure 3.1B, Table 3.1). Importantly, a direct comparison revealed that the TADV-A mosquitoes are significantly ($p < 0.001$) more effective as homozygotes, and similarly effective as heterozygotes, at blocking DENV infection as *Wolbachia*-infected mosquitoes.

3.4.5 Transgene impact on fitness

To determine whether the anti-DENV 1C19 scFv had any significant fitness effects on transgenic mosquitoes, we assessed several fitness parameters including larval to pupal development time, male and female fecundity and fertility, male mating success, and longevity (Table 2). No significant differences were observed between WT and TADV-A mosquitoes when examining male mating success and fecundity and fertility in both males and females ($p > 0.05$). However, we noticed a significant difference in larval to pupal development times ($p < 0.0001$), with WT mosquitoes developing, on average, 0.8 days faster than TADV-A mosquitoes. When assessing mosquito survivorship, there was no significant difference between WT and TADV-A males ($p > 0.05$; Figure S3.4), though WT female mosquitoes lived, on average, 4.5 days longer than TADV-A females ($p < 0.05$; Figure S3.4). The longevity of infected mosquitoes was also assessed. Transgenic, WT, or *w*Mel mosquitoes were infected with four DENV serotypes and their survivorship was assessed 14 dpi (Table 2). No significant ($p > 0.01$) differences between WT and TADV-A longevity upon infection with serotypes DENV-2, -3, and -4 were observed. However, there was a significant difference in survival upon infection with serotype DENV-1, with a higher proportion of WT mosquitoes surviving at 14 dpi ($p < 0.01$; Table 3.2, Figure S3.4). In addition, a significant difference in survival between *w*Mel mosquitoes and WT and TADV-A mosquitoes infected with serotype DENV-2 was observed ($p < 0.0001$; Figure S3.4).

3.5 Discussion

Our results demonstrate that conditional expression of the anti-DENV 1C19 scFv renders mosquitoes refractory to all four major DENV serotypes and therefore appears to be a potent viral inhibition strategy. While mosquitoes homozygous for the anti-DENV 1C19 scFv showed

complete refractoriness to DENV infection, heterozygous mosquitoes were still partially refractory to DENV infection, dissemination, and transmission, with significant, several orders-of-magnitude reductions in viral titers in the saliva. Given previous characterizations of the 1C19 scFv antibody, we presume that it achieves this refractoriness because, when it is secreted into the epithelium of the posterior midgut in mosquitoes (Edwards et al. 2000), it binds to the exposed fusion loop of DENV and inhibits the virus particle from releasing its genome into the cytoplasm for replication. Based on previous findings, it is likely that this significant reduction in viral titers would be sufficient to render heterozygous mosquitoes unable to transmit DENV to a susceptible host (Ferguson et al. 2015). Though this remains to be demonstrated, our results show that heterozygous 1C19 scFv antibody-expressing transgenic mosquitoes are just as efficient at viral suppression as—and homozygous mosquitoes are significantly more efficient than—*Wolbachia*-infected mosquitoes, which are currently being released for DENV control because they are known to be refractory to DENV (O'Neill 2018).

The difference in refractory levels in the homozygous versus heterozygous mosquitoes also suggests that the refractory phenotype is particularly sensitive to scFv antibody expression levels, a phenomenon previously observed with anti-malarial scFv transgenes (Isaacs et al. 2012) and anti-ZIKV synthetic small RNA transgenes (Buchman et al. 2019). If this means that complete refractoriness is susceptible to positional effects, e.g., not refractory when the scFv antibody transgene is expressed from a different, possibly more weakly expressing genomic insertion position, the identification of more robust midgut-specific promoters may help to ensure sufficiently high expression levels from a single copy of the transgene regardless of insertion site, as can the use of multiple anti-DENV scFv antibodies in a single transgene (Isaacs et al. 2012, 2011). Additionally, while we observed no significant reduction in multiple fitness parameters in

transgenic mosquitoes when compared to WT, some differences in fitness were observed, and more extensive analyses on fitness of both infected and uninfected transgenic heterozygotes and homozygotes (possibly after introgression with a field-collected mosquito strain) would have to be performed before use of such mosquitoes in the field.

The strategy we describe here provides an efficient "cargo" gene that can be coupled with a gene-drive system to reduce or eliminate the risk of DENV transmission by mosquitoes. In fact, previous efforts have demonstrated effective Cas9-mediated homing-based gene drives in malaria vectors (Gantz et al. 2015; Hammond et al. 2016; Kyrou et al. 2018), and even *Ae. aegypti* (Li et al. 2019). Additionally, since homing-based drive systems quickly convert heterozygotes to homozygotes (Champer, Buchman, and Akbari 2016), linking the anti-DENV 1C19 scFv antibody described here to such a drive system could, in theory, rapidly convert wild mosquito populations into transgenic homozygotes that would be completely resistant to DENV transmission. Of paramount importance to the viability of such an approach is the evolutionary stability of the refractory transgene, specifically in terms of the likelihood of viral-resistant evolution. Indeed, several studies have shown that, in some contexts, DENV can rapidly evolve resistance in response to neutralizing antibodies (Zou et al. 2012; Lai et al. 2007), and this may be especially likely in the TADV-A heterozygotes described in this study. However, this potentially can be managed through the selection of antibodies with mechanisms/epitopes that minimize the chance of evolved resistance and the use of a combination of distinct anti-DENV antibodies (many of which have been described, e.g., (Zou et al. 2012; Yamanaka, Kotaki, and Konishi 2013; Budigi et al. 2018; Lai et al. 2007; Smith et al. 2013)), as well as a combination of antibody and non-antibody based DENV refractoriness transgenes (e.g., (Franz et al. 2006; Mathur et al. 2010); (Yen et al. 2018); (Jupatanakul et al. 2017))). The deployment of such a pan-serotype-targeting strategy could serve

as an effective component of a comprehensive program to reduce the incidence and impact of DENV.

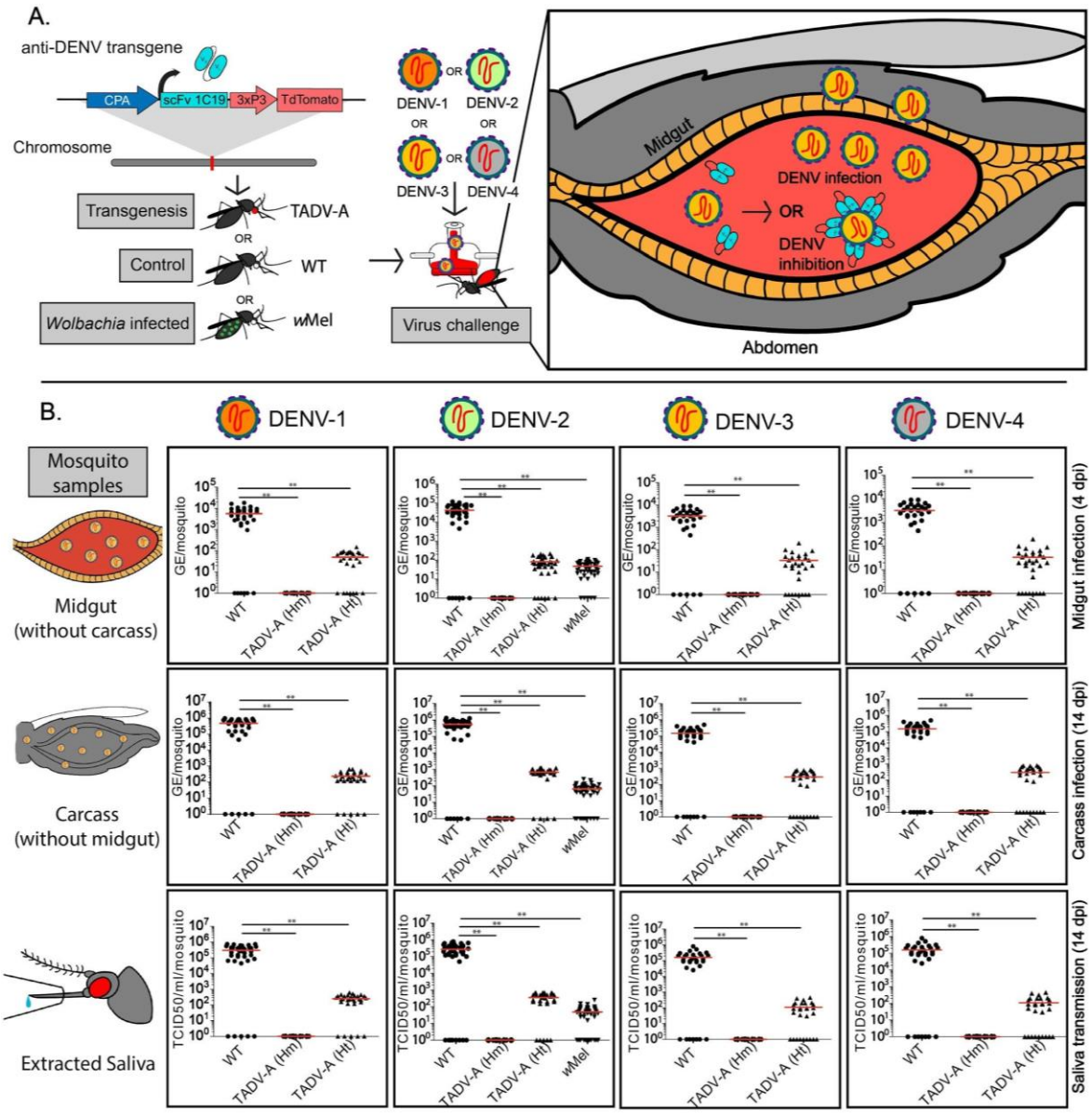
Due to similarities within viral families, this research could have far-reaching consequences for rendering mosquitoes resistant to other arboviruses like ZIKV and CHIKV by using similar genetic engineering strategies to develop scFv-based transgenes. Multiple potent antibodies that effectively neutralize these various mosquito-borne viruses have also been identified in the last decade (Long et al. 2019; Sun, Chen, and Lai 2017; Fernandez et al. 2018; Smith et al. 2015; Goo et al. 2019). Although not all of these will confer robust viral resistance when expressed *in vivo* in mosquitoes, the availability of diverse, well-characterized antibodies of this sort, largely as a result of antibody therapeutic development efforts (Sun, Chen, and Lai 2017), should allow for the identification of those that function within the desired context. Given the increasing incidence of disease caused by these viruses and the resulting global health implications, such scFv-based transgenes coupled with gene-drive systems (Li et al. 2019) can provide an effective, sustainable, and comprehensive strategy for reducing the impact of arboviral mosquito-borne diseases.

3.6 Acknowledgements

This chapter, in full, is a reprint of the material as it appears in PLoS Pathogens. Anna Buchman[1], Stephanie Gamez[1], Ming Li, Igor Antoshechkin, Hsing-Han Li, Hsin-Wei Wang, Chun Hong Chen, Melissa J. Klein, Jean-Bernard Duchemin, James E. Crowe, Jr., Prasad N. Paradkar, and Omar S. Akbari. "Broad Dengue Neutralization in Mosquitoes Expressing an Engineered Antibody". PLoS Pathogens. (2020). [[1] Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

3.7 Figures

Figure 3.1: Effect of anti-dengue virus (DENV) single-chain variable fragment (scFv) on DENV titers of TADV-A, *Wolbachia*-infected (wMel), and wildtype (WT) mosquitoes.
(A) Schematic of experiment. TADV-A mosquitoes were generated via transgenesis with the anti-DENV construct, and TADV-A, wMel, and WT mosquitoes were then challenged with a blood meal infected with one of four DENV serotypes (DENV-1, isolate ET243; DENV-2, isolate ET300; DENV-3, isolate ET209; or DENV-4, isolate ET288). After the infected blood meal enters the mosquito midgut, there are two potential outcomes: in the first (applies for all tested strains), the virus replicates and disseminates past the midgut to become transmissible; in the second (applies to TADV-A mosquitoes), the anti-DENV transgene expresses scFv antibodies in the midgut that bind to the virus and neutralize it. (B) Plots depicting viral titers. To determine if the anti-DENV transgene confers resistance to all four DENV serotypes, we determined viral titers in extracted midguts, carcasses, and saliva from WT, TADV-A (homozygous [Hm] and heterozygous [Ht]), and wMel infected mosquitoes. Viral genome equivalents (GE) from mosquito midguts (at 4 days post infection [dpi]) and carcass (at 14 dpi) were determined using RT-qPCR and calculated using previously published methods. Viral titers in the saliva were determined using the median tissue culture infective dose (TCID50) on Vero cells. For each experiment, data from three replicates is pooled. Red horizontal bars represent the mean GE/viral titer. **$p < 0.001$.


A.
anti-DENV transgene
CPA
scFv 1C19
3xP3
TdTomato
Chromosome
Transgenesis
TADV-A
OR
Control
WT
OR
Wolbachia infected
wMel
DENV-1
OR
DENV-2
OR
OR
DENV-3
OR
DENV-4
Virus challenge
Midgut
DENV infection
OR
DENV inhibition
Abdomen
B.
DENV-1
DENV-2
DENV-3
DENV-4
Mosquito samples
Midgut (without carcass)
Carcass (without midgut)
Extracted Saliva
GE/mosquito
TCID50/ml/mosquito
WT
TADV-A (Hm)
TADV-A (Ht)
wMel
Midgut infection (4 dpi)
Carcass infection (14 dpi)
Saliva transmission (14 dpi)

3.8 Tables

Table 3.1: Anti-DENV scFv effect on DENV infection, dissemination, and transmission rates. DENV titers in WT, heterozygous and homozygous TADV-A (TADV-A_{Ht} and TADV-A_{Hm}, respectively), and *w*Mel mosquitoes following a blood meal infected with one of four DENV serotypes are shown. DENV GE from mosquito midguts (at 4 or 14 dpi) and carcasses (14 dpi) of WT, TADV-A, and *w*Mel (for DENV-2 only) mosquitoes were determined using RT-qPCR and calculated using previously published methods. Viral titers in saliva collected from WT, TADV-A, and *w*Mel mosquitoes at 14 dpi were determined using $TCID_{50}$ on Vero cells.

DENV GE or viral titer on indicated dpi from specified tissue (proportion of mosquitoes with detected virus ; percentage of mosquitoes with detected virus)					
DENV Serotype	**Strain**	**4 dpi**	**14 dpi**		
		Midgut– GE	**Midgut– GE**	**Carcass– GE**	**Saliva– viral titer**
DENV-1	WT	5.70×10^3 (29/35; 83%)	3.80×10^5 (35/40; 88%)	4.80×10^5 (35/40; 88%)	3.04×10^5 (35/40; 88%)
	TADV-A_{Ht}	5.00×10^1 (20/28; 71%)	3.70×10^2 (25/30; 83%)	2.40×10^2 (25/30; 83%)	2.50×10^2 (25/30; 83%)
	TADV-A_{Hm}	0.00×10^1 (0/28; 0%)	0.00×10^1 (0/28; 0%)	0.00×10^1 (0/28; 0%)	0.00×10^1 (0/28; 0%)
DENV-2	WT	4.25×10^4 (34/40; 85%)	4.40×10^5 (40/46; 87%)	5.35×10^5 (40/46; 87%)	2.70×10^5 (38/46; 83%)
	TADV-A_{Ht}	8.20×10^1 (35/41; 85.4%)	3.90×10^2 (26/30; 86.6%)	6.70×10^2 (26/30; 87%)	3.56×10^2 (25/30; 83.3%)
	*w*Mel	4.75×10^1 (43/48; 90%)	5.10×10^1 (38/48; 79%)	6.45×10^1 (38/48; 79%)	4.90×10^1 (35/48; 73%)
	TADV-A_{Hm}	0.00×10^1 (0/35; 0%)	0.00×10^1 (0/30; 0%)	0.00×10^1 (0/30; 0%)	0.00×10^1 (0/30; 0%)
DENV-3	WT	1.80×10^4 (23/30; 77%)	2.90×10^5 (29/35; 83%)	3.50×10^5 (29/35; 83%)	2.90×10^5 (29/35; 83%)
	TADV-A_{Ht}	3.60×10^1 (22/30; 73%)	1.58×10^2 (20/30; 66%)	1.60×10^2 (20/30; 67%)	1.33×10^2 (20/30; 67%)
	TADV-A_{Hm}	0.00×10^1 (0/30; 0%)	0.00×10^1 (0/30; 0%)	0.00×10^1 (0/30; 0%)	0.00×10^1 (0/30; 0%)
DENV-4	WT	3.25×10^3 (25/30; 83%)	3.80×10^4 (25/32; 78%)	1.50×10^5 (25/32; 78%)	1.60×10^5 (25/32; 78%)
	TADV-A_{Ht}	3.40×10^1 (22/30; 73%)	2.38×10^2 (19/28; 68%)	2.95×10^2 (19/28; 68%)	1.08×10^2 (19/28; 68%)
	TADV-A_{Hm}	0.00×10^1 (0/27; 0%)	0.00×10^1 (0/28; 0%)	0.00×10^1 (0/28; 0%)	0.00×10^1 (0/28; 0%)

Table 3.2: Effect of anti-DENV scFv on fitness. Comparisons of several fitness parameters (left-most column) between WT (second column from left) and TADV-A mosquitoes (third column from left) suggest that there are few significant differences (right-most column) between the two groups, indicating that the anti-DENV scFv does not have a major impact on mosquito fitness. The survivorship of infected and non-infected mosquitoes is also shown. The median survival in days was determined for non-infected mosquitoes, and the percent of surviving mosquitoes separately infected by four DENV serotypes was assessed at 14 dpi.

Fitness Parameter	Strain: WT (N)	Strain: TADV-A (N)	*P*-value
Female fecundity[θ†§]	103.6 ± 3.8 (60; 6,213)	110.2 ± 4.4 (57; 5,756)	0.2578
Egg hatchability[θl§]	67.5 ± 3.2 (60; 4,208)	61.0 ± 4.1 (57; 4,046)	0.2149
Male mating success[θo¶]	93.00 ± 0.04 (43)	95.00 ± 0.04 (37)	0.7756
Male fecundity[θl§]	226.3 ± 15.7 (43; 9,730)	202.7 ± 17.2 (37; 7,318)	0.3141
Egg hatchability[θIII§]	75.9 ± 4.5 (43; 7,558)	73.1 ± 3.9 (37; 5,624)	0.6260
Larval to pupal development in days[θ§]	6.70 ± 0.77 (1,322)	7.50 ± 0.09 (774)	<0.0001
Female median survival in days[††]	53 (122)	48.5 (128)	0.0129
Male median survival in days[††]	14 (175)	14 (184)	0.1781
% Survival at 14 dpi with DENV-1[‡††]	80.8 (26)	43.5 (23)	0.0086
% Survival at 14 dpi with DENV-2[‡††]	72.7 (33)	69.2 (34)	0.6891
% Survival at 14 dpi with DENV-3[‡††]	64.9 (37)	52.2 (46)	0.2679
% Survival at 14 dpi with DENV-4[‡††]	41.3 (138)	48.8 (41)	0.7256

[θ]Mean ± SEM reported.

[†]Average number of eggs laid per female (Number of females scored; total number of eggs counted).

[l]Percentage of laid eggs that produced larvae (Number of females scored; total number of larvae counted).

[o]Percentage of single male outcrosses that gave rise to viable progeny.

[l]Average number of eggs laid per single male outcross (Number of male outcrosses scored; total number of eggs counted).

[III]Percentage of laid eggs that produced larvae per single male outcross (Number of male outcrosses scored; total number of larvae counted).

[§]Unpaired t test with Welch's correction was used.

[¶]Unpaired t test was used to evaluate the statistical significance between the proportions of fertile males.

[††]Mantel-Cox test was used.

[‡]Percentage of infected mosquitoes surviving at 14 dpi.

3.9 Supplemental Figures

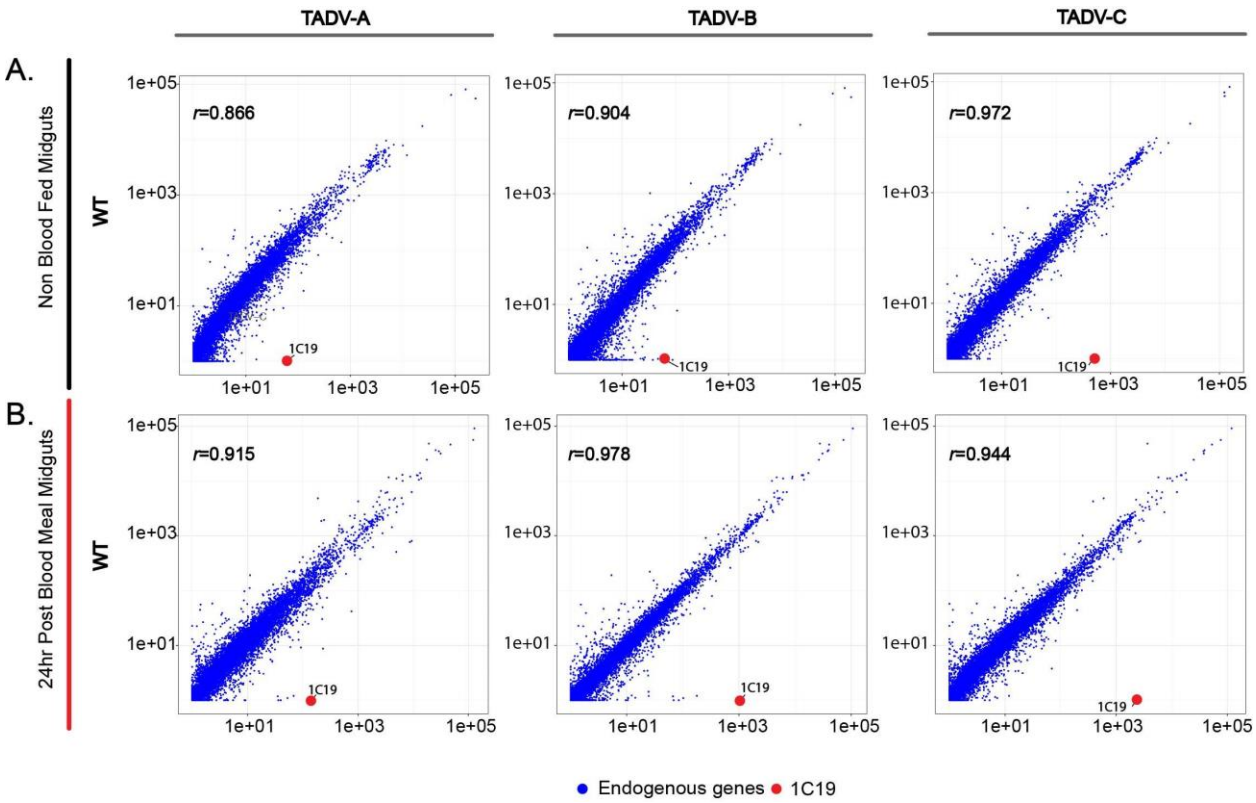


Figure S3.1: Expression correlation analyses of gene expression levels (indicated by TPM [transcripts per million] values) in dissected midgut tissues from WT or transgenic (TADV-A, -B, or -C) mosquitoes without a blood meal (A) and 24 hours after a blood meal (B). The y-axis corresponds to TPM values in WT samples, and the x-axis corresponds to TPM values in respective transgenic samples. Blue-colored points represent endogenous genes, and red-colored points represent the 1C19 scFv. Comparisons between WT and TADV samples suggest that 1C19 scFv expression is transgene-dependent and does not appear to significantly affect global expression levels of endogenous RNAs. Pearson correlation coefficients (*r*) between gene expression levels in WT versus transgenic samples are reported in bold in their respective graphs.

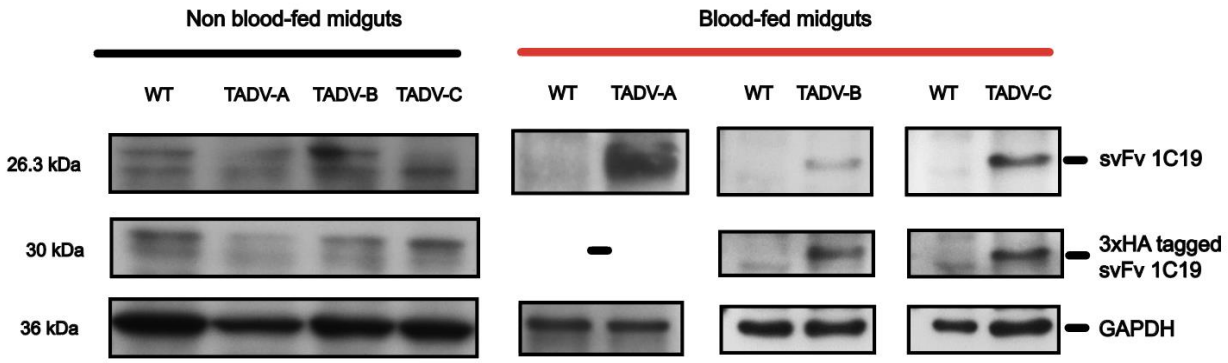


Figure S3.2: Western blot analyses to probe for the presence of the 1C19 scFv antibody protein in WT and transgenic midgut samples. Western blots were carried out utilizing a custom antibody specific for the 1C19 scFv protein, as well as an antibody to recognize the 3xHA tag, on dissected midgut tissues from non-blood-fed or 16-hour post-blood-meal WT or TADV-A, TADV-B, or TADV-C mosquitoes. An anti-GAPDH antibody was used as a control. The presence of a 26.3-kDa band confirms the expression of the 1C19 scFv protein in transgenic, but not in WT, mosquito midgut samples. The presence of a 30 kDa band indicates the presence of the 3xHA tag in TADV-B and TADV-C but not in WT or TADV-A mosquitoes.

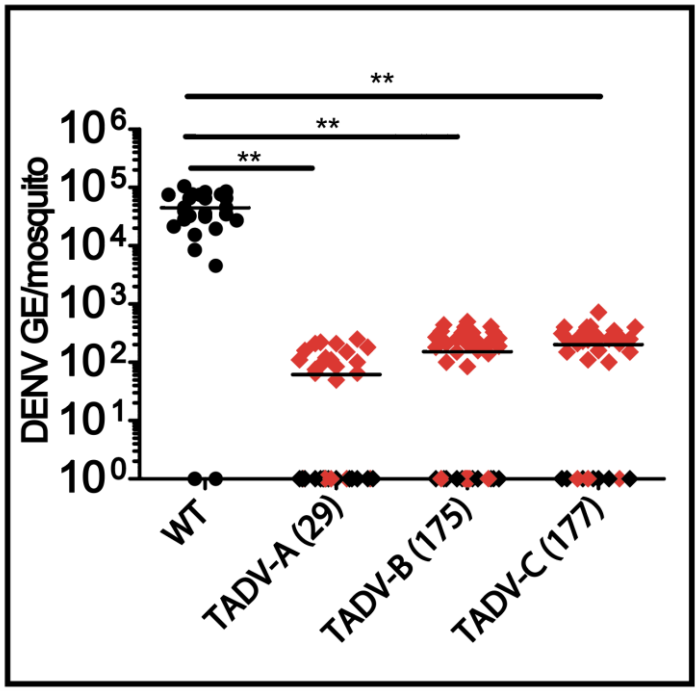
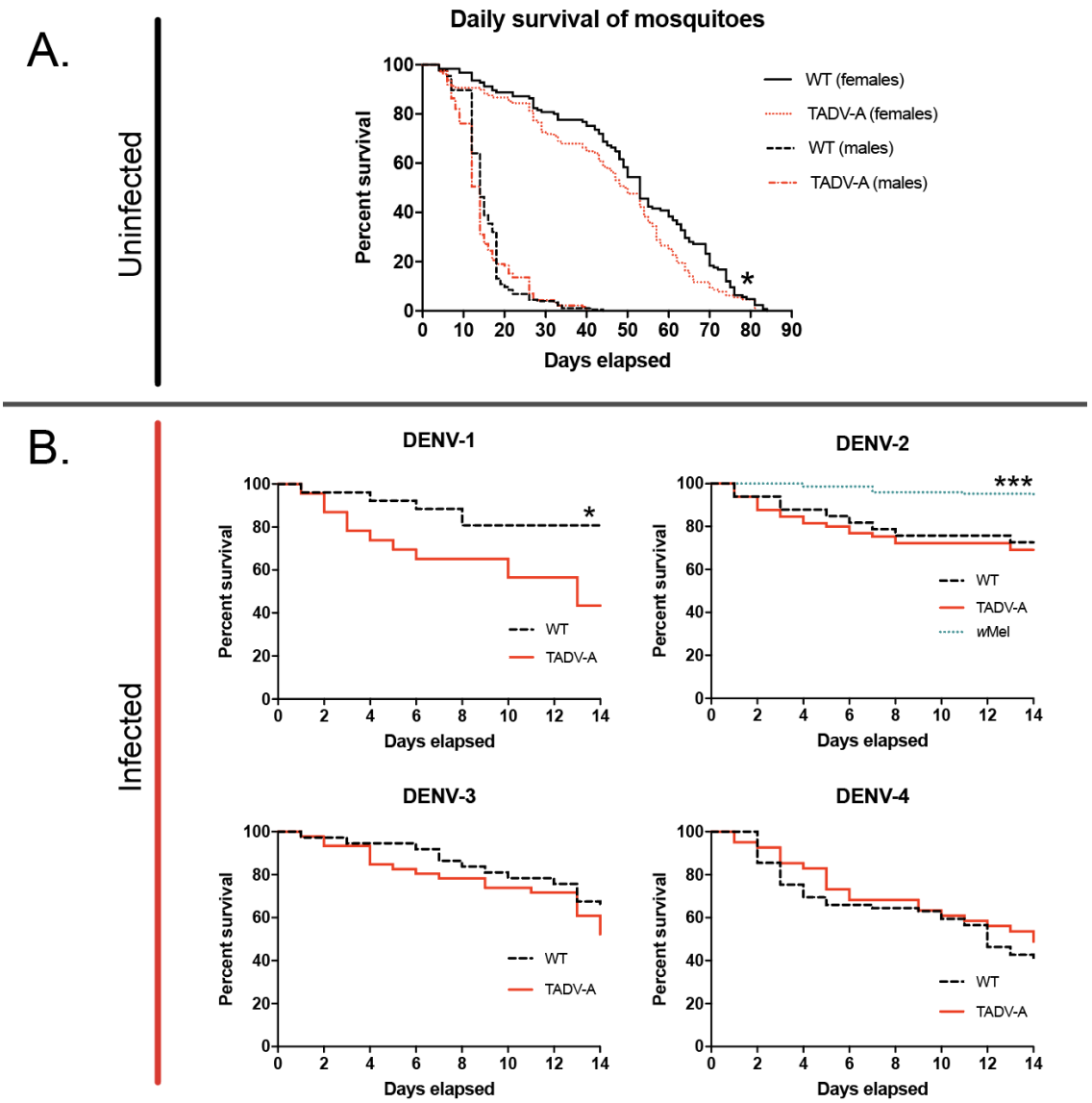


Figure S3.3: Effect of the anti-DENV scFv on DENV GE in three independent transgenic mosquito lines. DENV GE in WT and transgenic mosquito lines (TADV-A, TADV-B, or TADV-C) following a blood meal infected with DENV-2 (ET300 strain) are shown. DENV GE from mosquito midguts (at 4 dpi) of WT or transgenic mosquitoes were determined using real-time qPCR and calculated using previously published methods. Circles represent WT mosquitoes; black diamonds represent anti-DENV homozygous transgenic mosquitoes; red colored diamonds represent anti-DENV heterozygous transgenic mosquitoes. Horizontal bars represent the mean viral titer. The Mantel-Cox test was used for statistical analysis. $^{**}p < 0.001$.

Figure S3.4: Survivorship curves for uninfected WT and TADV-A mosquitoes and for DENV-infected WT, wMel, and TADV-A mosquitoes. The x-axis indicates the number of days elapsed after the start of the experiment, and the y-axis indicates the percent of mosquitoes surviving on each elapsed day. (A) For the uninfected panel, where the survivorship curves for WT and TADV-A male and female mosquitoes are shown separately, each line represents the accumulated results of 120–180 adult mosquitoes combined from 3 biological replicates. Significant differences in survivorship were observed between WT and TADV-A females, with WT females surviving, on average, 4.5 days longer ($p \leq 0.01$). (B) For the infected panels, WT, wMel, and TADV-A females were given a blood meal infected with DENV-1, DENV-2, DENV-3, or DENV-4 (as indicated on respective plot titles). The survivorship of infected mosquitoes was determined over the course of 14 days (the time it takes for the virus to disseminate past the midgut and eventually become transmissible). No significant differences in survivorship were found between WT and TADV-A mosquitoes when infected with DENV-3 and DENV-4, but significant differences were observed upon infection with DENV-1, with more WT mosquitoes (80%) surviving at 14 dpi than TADV-A mosquitoes (~40%; $p \leq 0.01$). When infected with DENV-2, more wMel mosquitoes (>90%) survived at 14 dpi compared to WT and TADV-A mosquitoes (both ~70%; $p < 0.0001$). The Mantel-Cox test was used to determine statistical significance. $*p \leq 0.01$, $***p < 0.0001$.


A.
Uninfected
Daily survival of mosquitoes
Percent survival
Days elapsed
WT (females)
TADV-A (females)
WT (males)
TADV-A (males)
*
B.
Infected
DENV-1
DENV-2
DENV-3
DENV-4
WT
TADV-A
wMel
*
***

3.10 Supplemental Tables

Table S3.1: Effect of the anti-DENV scFv on DENV-2 GE in three independent mosquito lines. DENV-2 GE are shown below for WT, TADV-A, TADV-B, and TADV-C mosquito lines following a blood meal infected with the DENV-2 ET300 strain. Midgut samples were collected 4 dpi, and GE were determined using real-time RT-qPCR and calculated using previously published methods.

Mosquito strain	GE of virus RNA/mosquito midgut at 4 dpi	Virus-positive mosquitoes/ Total mosquitoes (% infection rate)
WT	4.5×10^4	24/26 (92.3%)
TADV-A	6.1×10^1	17/37 (45.9%)
TADV-B	1.5×10^2	25/40 (62.5%)
TADV-C	2.0×10^2	25/35 (71.4%)

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Chapter 4: The developmental transcriptome of *Ae. albopictus*, a major worldwide human disease vector

4.1 Abstract

Aedes albopictus mosquitoes are important vectors for a number of human pathogens including the Zika, dengue, and chikungunya viruses. Capable of displacing *Aedes aegypti* populations, this mosquito adapts to cooler environments which increases its geographical range and transmission potential. There are limited control strategies for *Aedes albopictus* mosquitoes which is likely attributed to the lack of comprehensive biological studies on this emerging vector. To fill this void, here using RNA-seq we characterized *Aedes albopictus* mRNA expression profiles at 34 distinct time points throughout development providing the first high-resolution comprehensive view of the developmental transcriptome of this worldwide human disease vector. This enabled us to identify several patterns of shared gene expression among tissues as well as sex-specific expression patterns. To illuminate the similarities and differences with *Aedes aegypti*, a related human disease vector, we also performed a comparative analysis between the two developmental transcriptomes, identifying life stages where the two species exhibit similar and distinct gene expression patterns. These findings provide insights into the similarities and differences between *Aedes albopictus* and *Aedes aegypti* mosquito biology. In summary, the results generated from this study should form the basis for future investigations on the biology of *Aedes albopictus* and provide a gold mine resource for the development of transgene-based vector control strategies.

4.2 Introduction

Aedes albopictus (*Ae. albopictus*), the Asian tiger mosquito, is a medically important invasive species whose habitat range has significantly increased over the past 20 years (Benedict et al. 2007). Originally from East Asia and the islands of the Pacific and Indian Ocean, this species is now found in all continents except for Antarctica (Bonizzoni et al. 2013). The rapid expansion of *Ae. albopictus* has been attributed to its ecological plasticity, strong competitive aptitude, feeding behavior, vector competence, its ability to enter diapause to escape unfavorable seasonal conditions, and lack of effective control strategies (Reynolds et al. 2012). An increase in habitat range imposes a greater risk of transmitting several mosquito-borne pathogens such as Zika, chikungunya, and dengue virus (Shragai et al. 2017; Rezza 2012). Even though this species is considered a less efficient dengue vector than *Aedes aegypti* (*Ae. aegypti*), the primary vector of dengue virus and a closely related mosquito species, it is responsible for several outbreaks of dengue and chikungunya virus (Pagès et al. 2009; Vazeille et al. 2007; Ratsitorahina et al. 2008). Today both *Ae. albopictus* and *Ae. aegypti* are found in most Asian cities and in large parts of the Americas (Lambrechts et al. 2011). Both *Ae. albopictus* and *Ae. aegypti* feed on humans in daylight hours and rest indoors (Harrington, Edman, and Scott 2001; Dzul-Manzanilla et al. 2016; Valerio et al. 2010) and have similar larval niches, but their distributions depend on local environmental conditions (Kraemer et al. 2019). Interestingly, there are differences in dominance of mosquito vectors along urban-rural gradients. *Ae. albopictus* is often found in urban and rural environments, whereas *Ae. aegypti* tends to be an urban vector utilizing artificial containers (Kraemer et al. 2015; Tsuda et al. 2006). When both populations of mosquitoes are present in the same ecological niche *Ae. albopictus* mosquitoes tend to outcompete *Ae. aegypti* (Beilhe et al. 2012; O'meara et al. 1995). It is hypothesized that *Ae. albopictus* can do this because it is a superior larval competitor (Bagny

Beilhe et al. 2013). In addition, *Ae. albopictus* is found to be ecologically plastic where it can survive in cooler environments than *Ae. aegypti*, thus facilitating its spread to unconventional environments (Kraemer et al. 2019). To help combat this emerging mosquito, we need a better understanding of its biology to enable the innovation of effective control strategies.

Previously, a comprehensive developmental transcriptome study of *Ae. aegypti* was performed and provided insight into the complexity of the basic biology of these mosquitoes (Akbari, Antoshechkin, et al. 2013; Matthews et al. 2018). This enormous dataset has provided the community with a foundation of data enabling the functional characterization of novel genes and germline promoters (Akbari, Papathanos, et al. 2014; Li et al. 2017) which have subsequently been used to develop highly potent Cas9 endonuclease expressing strains (Akbari, Papathanos, et al. 2014), in addition to gene drives (Li et al. 2019). While diapause has been studied extensively for *Ae. albopictus* because of its importance to its survival in different environments (Diniz et al. 2017; Urbanski, Aruda, and Armbruster 2010; Urbanski et al. 2010; Reynolds et al. 2012), and the genome has been sequenced (Chen et al. 2015), there is currently no developmental transcriptome available for *Ae. albopictus*. Therefore to fill this void, here we provide a comprehensive analysis of the *Ae. albopictus* transcriptome throughout development which will provide the community with an invaluable resource to mine. In addition, this will provide a unique opportunity to perform comparative analysis and may even enable the discovery of novel genes and regulatory elements which may prove useful for innovating genetic control strategies.

4.3 Methods

4.3.1 Mosquito strain

Mosquitoes used for RNA extraction were from wildtype *Ae. albopictus* which originated from San Gabriel Valley, located in Los Angeles County, CA. Mosquito eggs were collected from oviposition traps set by the San Gabriel Valley Mosquito Control district. Collected eggs from sites were *Ae. albopictus* was known to circulate. These eggs were then hatched, checked for the characteristic stripe of *Ae. albopictus,* and reared for 10 generations before performing collection experiments. Mosquitoes were maintained in an insectary facility with a relative humidity of 70-80%, maintained at 28ºC, and with a 12 hr/12 hr light/dark cycle. Larvae were fed with ground fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany) and sex separated as pupae. Adults were maintained and fed with an aqueous solution of 10% sucrose. Females were blood-fed 3-5 days after eclosion on anesthetized mice. All animals were treated according to the Guide for the Care and Use of Laboratory Animals as recommended by the National Institutes of Health.

4.3.2 Total RNA isolation

In order to obtain a preliminary overview of the development of *Aedes albopictus*, one replicate of each sample was flash-frozen at specific time points, and total RNA was extracted using the Ambion mirVana mRNA isolation kit (Ambion/Applied Biosystems, Austin, TX). The number of tissues used per sample can be found in Table S4.1. The total RNA for a second testes replicate was extracted using the Qiagen RNeasy Mini kit (Qiagen, Germantown, MD). All sample collections were staged in the incubator at a relative humidity of 70–80%, 28ºC with a 12-hr/12-hr light cycle until the desired time point was reached. Samples were then immediately flash frozen. The adult non-blood fed (NBF) carcass was processed at 3 d after eclosion, and the adult

male carcass and testes were processed at 4 d after eclosion. After extraction, RNA was treated with Ambion Turbo DNase (Ambion/Applied Biosystems, Austin, TX). RNA integrity was assessed using RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513).

4.3.3 Illumina sequencing

RNA-seq libraries were constructed using NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770) following the manufacturer's instructions. Briefly, mRNA isolated from ~1 μg of total RNA was fragmented to an average size of 200 nt by incubating at 94 °C for 15 min in first strand buffer, cDNA was synthesized using random primers and ProtoScript II Reverse Transcriptase followed by second strand synthesis using NEB Second Strand Synthesis Enzyme Mix. Resulting DNA fragments were end-repaired, dA tailed and ligated to NEBNext hairpin adaptors (NEB #E7335). After ligation, adaptors were converted to the 'Y' shape by treating with USER enzyme and DNA fragments were size selected using Agencourt AMPure XP beads (Beckman Coulter #A63880) to generate fragment sizes between 250 and 350 bp. Adaptor-ligated DNA was PCR amplified followed by AMPure XP bead clean up. Libraries were quantified with Qubit dsDNA HS Kit (ThermoFisher Scientific #Q32854) and the size distribution was confirmed with High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies #5067- 4626). Libraries were sequenced on Illumina HiSeq2500 in single read mode, with an approximately similar depth of 30 million reads per sample, and a read length of 50 nt following manufacturer's instructions. Base calls were performed with RTA 1.18.64 followed by conversion to FASTQ with bcl2fastq 1.8.4.

4.3.4 Poly(A+) read alignment and quantification

The *Ae. albopictus* reference genome assemblies and gene models were retrieved from NCBI (canu_80X_arrow2.2, GCA_001876365.2 for genome assembly and ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/001/876/365/GCF_001876365.2_canu_80X_arrow2.2/GCF_001876365.2_canu_80X_arrow2.2_genomic.gff.gz for gene models) (Table S4.2). Reads from RNA-seq libraries were aligned to the *Ae. albopictus* genome using STAR aligner (Dobin et al. 2013) with default parameters with the addition of '--outFilterType BySJout' filtering option and '--sjdbGTFfile GCF_001876365.2_canu_80X_arrow2.2_genomic.gtf' GTF file. Gene models were quantified with featureCounts (Liao, Smyth, and Shi 2014) using '-t exon -g gene_id -M --fraction' options. TPM (Transcripts Per Million) and FPKM (Fragments Per Kilobase Million) values were calculated from count data using Perl script addTpmFpkmToFeatureCounts.pl (Script S4.1). For depiction of data, TPM was chosen because it considers the combined effects of sequencing depth and gene length for the read counts and is a commonly used metrics for cross sample comparisons. Quality assessment of the data included calculating Pearson correlations, hierarchical clustering (measure: Euclidean distance; clusters: average linkage), and principal component analyses (PCA) between samples.

4.3.5 Use of DESeq2 for the exploration of data

To obtain insights into the types of genes upregulated in sex-specific samples, we performed a DESeq2 analysis in R using counts from libraries from each sex (Love, Huber, and Anders 2014). The count data were imported into the DESeq2 framework and analyzed with default parameters. Due to the single replicate (with the exception of the male testes) collection of

samples in our study, we cannot accurately identify differential expression among samples. This will only allow us to explore our data by obtaining fold-changes and identifying potential genes that are upregulated. In our sex-specific analyses, we further sorted genes with fold changes >20x.

4.3.6 Clustering and Gene Ontology (GO) analysis

TPM values produced by featureCounts for 47 RNA-seq libraries were clustered using Mfuzz R software package (Kumar and E Futschik 2007). Mfuzz uses fuzzy c-means algorithm to perform soft clustering, which allows cluster overlap and has been demonstrated to perform favorably on gene expression data. The resulting clusters were analyzed for overrepresentation of GO terms using a hypergeometric test implemented using the GOstats R software package (Falcon and Gentleman 2007). Pfam domains for the *Ae. Albopictus* gene set were identified by running hmmscan (Finn, Clements, and Eddy 2011) and associated GO terms were added using pfam2go mapping downloaded from the Gene Ontology Consortium (The Gene Ontology Consortium and The Gene Ontology Consortium 2019). Hypergeometric tests were performed separately for biological process, molecular function, and cellular component ontologies. Only GO terms with a *p*-value < 0.05 were selected. Sample dendrograms and PCA plots were generated in R and plotted with ggdendro and ggplot2 packages (Kassambara 2015).

4.3.7 Comparative analysis between *Ae. albopictus* and *Ae. aegypti* transcriptomes

Orthologous gene pairs between *Ae. albopictus* and *Ae. aegypti* were identified using the best reciprocal BLAST hit approach. Briefly, protein sets for *Ae. albopictus* and *Ae. aegypti* were downloaded from NCBI: (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/001/876/365/GCF_001876365.2_canu_80X_arrow

2.2/GCF_001876365.2_canu_80X_arrow2.2_protein.faa.gz and ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/002/204/515/GCF_002204515.2_AaegL5.0/GCF_002204515.2_AaegL5.0_protein.faa.gz, respectively), blast databases for both sets were constructed with ncbi-blast v.2.7.1+ and blastp searches of each set against the other were performed with default parameters. Blast hits were parsed with a Perl script collectBlastHits.pl (Script S4.2) and the best hit for each query sequence in the other species was identified and retained as an ortholog only if it identified the original query sequence as the best hit when searched in the other direction using a Perl script findBestReciprocalHitWithScore.pl (Script S4.3). No explicit e-value cutoff was specified during searches. Protein IDs were then translated to gene IDs and multiple proteins per gene were collapsed to produce a nonredundant set of 10,696 orthologous gene pairs, which represents 54.51% and 27.62% of genes encoded by *Ae. aegypti* and *Ae. albopictus* genomes, respectively. The best reciprocal hit procedure required a strict one to one correspondence between *Ae. aegypti* and *Ae. albopictus* genes. Because of the extensive duplication known to exist in the current version of *Ae. albopictus* genome (predicted to encode 38,719 genes compared to 19,623 in *Ae. aegypti*), many genes produced ambiguous mapping and had to be eliminated from the list of orthologs. Nevertheless, the ortholog set identified here faithfully captures the general trends of the developmental transcriptome as illustrated by very similar sample clustering patterns based on expression values of orthologs alone and of the full gene set (Figure 4.2 and 4.3). To quantify expression values of orthologous genes, RNA-seq reads from the *Ae. aegypti* and *Ae. albopictus* samples were aligned to corresponding genomes using STAR (i.e. *Ae. aegypti* reads were mapped to *Ae. aegypti* genome, *Ae. albopictus* reads were mapped to *Ae. albopictus* genome) with default parameters with the addition of '--outFilterType BySJout' filtering option. Gene counts were extracted with featureCounts using '-t exon -g gene_id -M --fraction' options and complete

species-specific GTF files. Count data for orthologous gene pairs were parsed from full featureCount tables and used to identify the fold-change and base mean between the two species for each developmental stage using DESeq2. DESeq2 was run with default settings. MA plots for each sample pair were generated with ggplot2.

4.3.8 Data availability

Ae. Albopictus mosquito line is available upon request. All sequencing data has been made publically available at NCBI Sequence Read Archive (SRA) under Accession number (BioProject ID (PRJNA563095)).

4.4 Results

4.4.1 *Ae. albopictus* developmental transcriptome timepoints

To establish a comprehensive global view of gene expression dynamics throughout *Ae. albopictus* development, we performed Illumina RNA sequencing (RNA-Seq) on one replicate for each of the 47 unique samples representing 34 distinct stages of development (Figure S4.1A). These time points incorporated 31 whole animal and 16 tissue/carcass samples. For example, for embryogenesis 19 samples were collected; the first three time points, 0-1 hr, 0-4 hr, and 4-8 hr embryos, capture the maternal-zygotic transition, whereas 16 additional embryo samples were collected at 4 hr intervals until 72 hr to capture the duration of embryogenesis. Samples from four larval stages (instars 1-4) and sex-separated early and late male and female pupae were collected to capture the aquatic life cycle. Additionally, whole dissected ovaries and carcasses (whole female bodies lacking ovaries) from NBF females and from females at 12 hr, 24 hr, 36 hr, 48 hr, 60 hr, and 72 hr post-blood meal (PBM) were collected to examine the pre-vitellogenic “resting stage”

through the completion of oogenesis. Adult male testes and carcasses (lacking testes) were collected at four days post eclosion to investigate male-specific germline and somatic gene expression.

To achieve single nucleotide resolution, we extracted total RNA from each sample and sequenced entire transcriptomes using the Illumina HiSeq2500, and generated 1.56 billion 50nt reads corresponding to total sequence output of 78.19 GB with close to 95% of the reads aligning to the most contiguous and complete *Ae. albopictus* assembly available (assembly: canu_80X_arrow2.2, strain: C6/36, VectorBase) (Figure S4.1A; Tables S4.1, S4.2, S4.3). On average, 33,271,957 Illumina sequencing reads were obtained per sample/library (Table S4.4). A previous developmental transcriptome alignment using *Ae. aegypti samples* showed up to 93% of uniquely mapped reads (Akbari, Antoshechkin, et al. 2013). We anticipated similar results for *Ae. albopictus*, however, we found about 45% of reads uniquely mapped (Figure S4.1A; Table S4.3). This low percentage likely reflects the high duplication of the current *Ae. albopictus* genome assembly.

4.4.2 Global transcriptome dynamics

To capture the global dynamics of gene expression, we quantified the gene expression profiles across all developmental timepoints (Figure 4.1; Tables S4.4 and S4.5). Because this study is a developmental time course, the correlation between adjacent samples in the time course provide a reference to whether a sample is an outlier. According to Table S4.6, the correlation values between adjacent samples are extremely high as expected. The only exception was the male testes sample and for this we performed a second replicate to confirm our results. In general, the number of expressed genes (FPKM>1) gradually increases through embryogenesis, reaching a

peak at 68-72 hr (Figure S4.1B). This likely reflects the embryo developing and preparing for the next major developmental stage, the larval stage. In this stage, the number of genes expressed are fewer in 1st larval instar, but then increases in the subsequent 2nd to 4th instars. After the larval stage, the animal undergoes metamorphosis into pupae where sexual dimorphism is apparent. During the early pupal stages, the number of genes increases suggesting transcripts involved in hormone production and initiation of adult formation are being expressed (Margam, Gelman, and Palli 2006). In the adult stages the difference between the male and female germline is obvious with males expressing the highest number of genes. When females take a blood meal for egg production, the number of genes expressed in the ovary do not seem to vary notably, however when looking at their corresponding carcasses, varying levels across PBM females are observed suggesting dynamic gene expression in somatic tissues (Figure S4.1B). Interestingly, the tissue with the highest number of genes expressed corresponds to the male testes. In contrast, the lowest number of expressed genes correspond to the 24 hr PBM female carcasses. Analysis of pairwise correlations revealed that almost every developmental stage is most highly correlated with its adjacent stage and this is particularly evident during embryogenesis (Figure 4.1A). Notable exceptions to this trend occur in 24-36 hr PBM female carcass and 36-48 hr PBM ovaries, suggesting that these represent important points where physiological transitions occur in blood-fed females. Diapause samples (0 through 4 weeks) and NBF and 12 hr PBM ovaries are highly correlated with the mid-stages of embryogenesis suggesting similar genes are expressed in these samples. In the 0-1 hr embryo time point, we see similar gene expression with the 60- and 72- hr PBM ovaries likely reflective of maternally deposited transcripts. Samples with unique gene expression include the male germline, 24 hr PBM female carcass, 24 hr and 36 hr PBM ovaries, and late pupae (Figure 4.1A).

To further visualize the various patterns of gene expression and the relationships between the samples, hierarchical clustering and PCA analyses was performed (Figure 4.1B, C, D). Based on these analysis, embryo, PBM ovaries, pupae, larvae, and PBM female carcass samples tend to cluster closer together which is expected because their gene expression profiles are similar as these are developmentally related samples. Interestingly however, in Figure 4.1D, the male testes sample clusters away from all other samples, reflecting a distinguishing difference between this sample as compared to other samples sequenced. To observe patterns of co-regulated gene expression we used a soft clustering algorithm and identified 20 distinct patterns that included 543 to 2760 genes (Figure 4.2A). Each cluster in Figure 4.2A contains a set of *Ae. albopictus* genes that have an assigned membership value to indicate the degree of similarity to genes in that cluster (Table S4.7). The majority of these clustering patterns correspond to the developmental stages and transitions of the mosquito. For example, clusters 1 through 7 include genes that are associated with embryogenesis (Figure 4.2A). To investigate the functional associations of the genes in each cluster, we performed gene ontology (GO) analysis and focused on gene descriptions with *p*-values<0.05 (Table S4.8). Here, we will focus on listing gene descriptions that are relevant to the mosquito's developmental stage. Genes in clusters 1 through 7 are highly enriched in genes involving nucleic acid binding (e.g. LOC109417994, LOC109422308 and LOC109424406), organic cyclic compound binding in the molecular function category. In the biological processes category, some highly enriched genes include macromolecule catabolic, metabolic, and biosynthetic processes (e.g. LOC109410609, LOC109410731 and LOC109429162), DNA-templated transcription, and regulation of nitrogen compound metabolic processes (Table S4.8). These processes correspond to the necessities of the developing embryo as it transitions between stages with rapidly changing demands. Energy is supplied to the embryo through the breakdown

of biomolecules. After embryogenesis, the deposited egg can enter diapause, a dormant state that allows the mosquito embryo to survive unfavorable conditions (Armbruster 2016). Cluster 8 includes gene expression required for diapause in dormant embryos. Here, several biological processes such as translation, amide biosynthetic, peptide biosynthetic and metabolic processes are enriched as well as molecular function terms associated with lipid transporter activity, lipid-A-disaccharide synthase activity, and ribonucleoside binding (Table S4.8). Genes enriched in this cluster include several lipases (to name a few: LOC109417138, LOC109401099, LOC109430899, and LOC109430905), several fatty acid hydroxylases (to name a few: LOC109400137, LOC109432075, and LOC109397180), and some proteases (LOC109406257, LOC109411917, and LOC109402104) (Table S4.8). This is likely due to the expression of genes that correspond to specific metabolic events associated with diapause to enable cold tolerance (Diniz et al. 2017). When an embryo hatches under favorable conditions it then enters the larval life-stage which is composed of four separate larval stages (1-4) before the pupal stage. In our clustering analysis, the larval stages correspond to clusters 9 and 10 (Figure 4.2A). In both of these clusters genes are enriched for serine-type peptidase activity, chitin binding, metallopeptidase activity, oxidoreductase activity, and ATPase activity under the molecular function category. Biological processes taking place include proteolysis, amino sugar metabolic, chitin metabolic, glucosamine-containing compound metabolic, and amino sugar metabolic processes (Table S4.8). The metabolic processes are likely involved in preparing the larva to acquire the energy reserves that will be used for egg development (Telang et al. 2006). Following the larval stage, the mosquito then enters the pupal stage, the final aquatic stage in the mosquito's life cycle. Here, clusters 11, 12 and 15 correspond to the pupal stages and include terms in the molecular function category enriched for structural cuticular constituents, oxidoreductase, peptidase, and serine-type peptidase

activity, which are likely involved in immunity and the hydrolysis of nutrients (Saboia-Vahia et al. 2013). Steroid biosynthetic and metabolic processes are also enriched which suggests hormones like ecdysteroids, which are crucial for metamorphosis, are preparing the pupa to molt into an adult mosquito (Margam, Gelman, and Palli 2006).

Following the pupal stage, the aquatic life cycle ends with the animal undergoing metamorphosis into an adult. In males, the carcass and gonads have different terms enriched (clusters 13 for carcass and 14 for male testes). Cluster 13 include terms enriched for NADH dehydrogenase activity and oxidoreductase activity in the molecular function category. In the biological processes category, some terms enriched include several ribonucleotide and nucleotide metabolic processes (Table S4.8). In the male germline (cluster 14), some terms enriched in the molecular function category include microtubule motor activity, olfactory receptor activity, and neurotransmitter receptor activity. In the biological processes category, some terms enriched include spermatid development, microtubule based processes, sensory perception of chemical stimulus. Like *Anopheles gambiae* and *Ae. aegypti* mosquitoes, *Ae. albopictus* use chemosensation to activate their spermatozoa by modulating sperm activation and perhaps the orientation of spermatozoa (Pitts et al. 2014). Cluster 16 likely corresponds to the developing pupa and the developing germline of male and female pupae during the late pupal stages (Figure 4.2A, B). Here, there are several genes that peak in the late pupal samples, male testes, and late vitellogenic ovarian stage. Clusters 17, 18, and 19 correspond to ovarian development which consist of pre-vitellogenic (NBF ovaries) and vitellogenic (PBM ovaries) stages. In these ovarian developmental stages some genes that are enriched include cellular response to stimulus and Ras protein signal transduction which are important means of communication during the processes of oocyte and eggshell patterning (Dana et al. 2005). In addition, several metabolism processes that are crucial for the

breakdown of organic molecules such as deoxyribonucleotides are crucial to support cell division (Telang et al. 2013). Finally, cluster 20 corresponds to genes that are expressed in PBM female carcasses. In the biological processes category, some terms are enriched for lipid metabolic processes and sensory perceptions and are both related to the processes required to support the future developing zygote in the ovaries. In the molecular function category, some relevant terms enriched include those with serine-type endopeptidase activity, serine hydrolase activity, and endopeptidase activity which are likely important for breaking down blood proteins (Bian, Raikhel, and Zhu 2008).

4.4.3 Sex-biased gene expression overview

Genes expressed in the germline of males and females are believed to play important roles in evolution, contributing to reproductive fitness, isolation, and speciation (Whittle and Extavour 2017). Thus it is important to study the role of sex-biased gonad genes in evolution. To gain some insight into the sex-biased differences in *Ae. albopictus*, we compared the transcriptomes of male and female samples (male testes and carcass, female NBF and PBM ovaries and carcasses, and pupal samples). In order to identify sex-biased genes, we performed a pairwise comparison for each sex-specific stage using a limited DESeq2 approach. For identifying biased genes in either the male or female germline, a total of seven pairwise comparisons were done including: Male testes vs NBF ovaries; Male testes vs 12 hr PBM ovaries; Male testes vs 24 hr PBM ovaries; Male testes vs 36 hr PBM ovaries; Male testes vs 48 hr PBM ovaries; Male testes vs 60 hr PBM ovaries; Male testes vs 72 hr PBM ovaries. For identifying biased genes in either the male or female soma, a total of 8 pairwise comparisons were done including: Male carcass vs NBF carcass; Male carcass vs 12 hr PBM carcass; Male carcass vs 24 hr PBM carcass; Male carcass vs 36 hr PBM carcass;

Male carcass vs 48 hr PBM carcass; Male carcass vs 60 hr PBM carcass; Male carcass vs 72 hr PBM carcass; and Male pupae vs Female pupae. In these pairwise analyses, identification of sex-biased genes were accomplished by looking at genes that were upregulated in females (indicated by negative log2FoldChange values) and males (indicated by positive log2FoldChange values). Because of the lack of replicates in our dataset, we cannot unambiguously assign significance values to the comparison results, which are intended to represent exploratory analysis in order to get an overall impression of sex-biased expression patterns. Further experimental characterizations will be required to confirm the expression dynamics of the identified genes. After generating a list of up and down-regulated genes in each sample, we decided to focus our discussion on a subset of genes, which display >20x fold overexpression in one of the sexes. Minimal expression can be expected from the opposite sex.

4.4.3.1 Female-biased genes in NBF and PBM states

In our sex-biased comparison analysis, we found a combined total of 492 overexpressed genes that were upregulated in both the germline and somatic tissues of female mosquitoes and are listed in Table S4.9. Out of these genes, 128 have orthologs in *Ae. aegypti* while the rest seem to be specific to *Ae. albopictus*. This may be due to deficiencies in the annotation of the *Ae. albopictus* genome assembly or may represent loci that are specific to this species. Only 164 loci (out of 492) are uncharacterized. Additional work needs to be done to uncover the identity and function of these unknown loci that are highly expressed in the female ovaries. In NBF carcass samples, genes with >20x expression include 30kDa salivary gland allergens, trypsins, and several uncharacterized genes (Table S4.9).

Ingestion of vertebrate blood is essential for egg maturation by female mosquitoes. In order to gain insight into how a blood meal changes gene expression in female ovaries, we also analyzed PBM ovary and carcass samples (Table S4.9). A distinct pattern emerges when NBF females encounter a blood meal: genes expressed in NBF samples are downregulated in PBM samples and vice versa (Table S4.9). In PBM ovaries samples, vitelline genes become upregulated after a blood meal (beginning 12 hrs PBM) and start to decrease at 36 hrs PBM. Eventually their expression is downregulated to a point where almost no vitelline genes are sufficiently expressed (60 hr PBM). This may indicate a point where vitellogenesis halts and transitions to other oogenesis processes (V. A. Kokoza et al. 2001).

4.4.3.2 Male-biased genes

In performing our global expression analysis, we found that the *Ae. albopictus* male testes sample clustered very distantly when compared to all other time point samples (Figure 4.1C, D). To ensure this was not due to an error in sample preparation or single replicate basis, we collected a second male testes replicate and performed a correlation analysis against all samples (Table S4.6). We find that both testes replicates are highly similar (r = 0.903) suggesting that the initial findings are supported. In our sex-bias comparison analysis, we found a combined total of 485 genes that were >20x upregulated in both types of male tissues (Table S4.10). Out of these only 220 are orthologous to *Ae. aegypti* genes. Among the genes with the highest upregulation in the male germline include several uncharacterized loci, cytosol aminopeptidases, tubulin chains, a couple kinases, and a 36.4 kDa proline rich protein (Table S4.10). Other genes with relatively high prevalence in the testes include cilia- and flagella-associated proteins, cytochrome c oxidase subunits, dynein chains, and testis-specific serine/threonine protein kinases. It is likely that these genes are crucial for sperm development and management for male reproductive success. Among

these exclusive genes we note an interesting observation. Two genes, LOC109414298 and LOC109407232 are found in different locations (Locations NW_017856468.1: 340,363-342,022 and NW_017856205.1: 8,069,187-8,070,779, respectively) and have 100% amino acid similarity to *Ae. aegypti*'s beta tubulin 2 protein sequence (Smith et al. 2007). While the nucleic acid sequence differed between *Ae. aegypti* beta tubulin 2 and *Ae. albopictus* LOC109414298 and LOC109407232, their amino acid sequences were identical. This suggests that *Ae. albopictus* contains at least two copies of the beta-tubulin 2 gene as paralogs. This finding is supported by a previous finding where twice as many seminal fluid proteins were identified in *Ae. albopictus* compared to *Ae. aegypti* (Degner et al. 2019). While we note there is duplication present in our species, we do not yet fully understand how two copies of a gene can affect mosquito biology of *Ae. albopictus*.

4.4.4 Small RNA pathway protein dynamics

There are three major classes of regulatory small RNAs in animals that include: microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs). Small RNAs are classified based on their size and interaction with Argonaute proteins. In addition, they are each involved in regulating particular processes in the mosquito. For example, miRNAs are shown to post-transcriptionally regulate transcript levels and the translational status of mRNA (Lucas, Myles, and Raikhel 2013). In mosquitoes, some miRNAs have been implicated in the regulation and function of blood digestion and ovarian development (Lucas, Myles, and Raikhel 2013). In contrast, the siRNA pathway is responsible for modulating arbovirus replication and can be responsible for transposable element silencing. Finally, the piRNA pathway is suggested to control the remobilization of transposable elements and may take part in antiviral immunity. In

Aedes mosquitoes, there are seven PIWI proteins each with a seemingly distinct function (Miesen, Girardi, and van Rij 2015; Miesen, Joosten, and van Rij 2016). To gain a global view of genes involved in small RNA processing we generated a heatmap to visualize their expression across development (Figure S4.2; Table S4.11). Increased expression of two piRNA genes, mael (AALF019672) and gstf (AALF023639), were apparent in NBF and PBM ovaries (Figure S4.2). Interestingly, twin (AALF018294), a CCR4 deadenylase, is highly expressed when the embryos undergo diapause. In *Drosophila*, this gene is shown to promote the decay of specific mRNAs in the early embryo (Rouget et al. 2010). In *Ae. albopictus*, it may serve as a mechanism to ensure diapause is transcriptionally arrested until there is an environmental signal that induces diapause termination. In contrast to all the stages, the male and female NBF and PBM germline contained piwi2 and piwi3 to be highly expressed (Figure S4.2). While there are several functions for the use of piRNAs in the insect germline, it is likely this observation suggests that the PIWI pathway is involved in silencing retrotransposons (Kalmykova, Klenov, and Gvozdev 2005; S. H. Wang and Elgin 2011). Piwi3 was found to be downregulated in male testes, later PBM ovaries, and in the early embryo (0-1 hr and 0-4 hr) (Figure S4.2). While there is still not much known about each particular PIWI protein, a study found that piwi3 may be associated with viral dissemination in mosquitoes (Y. Wang et al. 2018).

4.4.5 Comparison between *Ae. albopictus* and *Ae. aegypti* development transcriptomes

We next sought to determine the similarities and differences between the developmental transcriptomes of *Ae. aegypti* and *Ae. albopictus* mosquitoes. To establish orthologues, we performed BLAST searches on the proteomes of both species and identified best reciprocal hits ensuring one-to-one relationship between genes in the two species resulting in 10,696 orthologous

pairs representing 54.51% and 27.63% of genes encoded by *Ae. aegypti* and *Ae. albopictus* genomes, respectively (Table S4.12 for best reciprocal hits). For BLAST searchers, no explicit e-value cutoff was specified during searches, but only 8 of 11,687 identified orthologous protein pairs (0.068%) had blastp e-value above 0.001 (0.37 the highest). 11,666 of orthologs (99.820%) had e-value below $1e^{-10}$; 10,353 (88.586%) had e-values below $1e^{-100}$; and 8,033 (68.734%) had e-values of 0 (Figure S4.3). Using previously published *Ae. aegypti* developmental transcriptome data (Akbari, Antoshechkin, et al. 2013), we conducted a set of analyses comparing gene expression levels between the two species' developmental stages with the aim to gain insight into possible differences in their biology. To determine expression values of orthologous gene pairs, RNA-seq reads from the *Ae. aegypti* and *Ae. albopictus* samples were aligned to corresponding genomes using STAR (i.e. *Ae. aegypti* reads were mapped to *Ae. aegypti* genome, *Ae. albopictus* were mapped to *Ae. albopictus* genome). The reads were quantified with featureCounts using species-specific GTFs defining orthologous gene models. On average, 57.6 % (range: 23.3% to 72.9%) and 34.6% (range: 29.1% to 39.4%) of RNA-seq reads from *Ae. aegypti* and *Ae. albopictus* datasets were mapped to orthologs, respectively (Table S4.13). Sample clustering and PCA analysis using expression values of orthologous genes revealed that the majority of the corresponding sample timepoints and tissues between both species display similar overall expression patterns and are found adjacent to each other (Figure 4.3A, 4.3B). A notable exception is the testes sample pair, which cluster far apart presumably reflecting considerably different gene expression programs. Finally, we calculated Pearson correlations between TPM values of the corresponding samples and then plotted them on a heatmap to confirm similarities between embryonic samples (Figure 4.3C, Figure S4.4, Table S4.14). Embryonic samples between species have higher correlations indicating that the genes involved in this developmental stage are very

similar or shared. This is also true for all other tissues/time points, however, with the exception of the male testes, consistent with the results of clustering analysis (Figure 4.3C; Figure S4.4; Table S4.14).

We next performed DESeq2 analysis between *Ae. albopictus* and *Ae. aegypti* samples at each developmental time point to gain some insight into the differences in expression of orthologous genes (Table S4.15). As with the sex-bias analysis, these comparisons are exploratory in nature due to the lack of replicates. They are aimed at high-level overview of orthologous gene expression in the two species. In the lack of replicates, few genes reach significant adjusted p-values due to the likely overestimation of dispersion of count values by the DESeq2 algorithm making it impossible to draw definitive conclusions about genes with poor p-values. The genes that satisfy the significance criterion however may represent good candidates for truly differentially expressed genes and may provide a foundation for more detailed analyses in the future. Here we will focus on five samples (NBF ovaries, female pupae, 4th instar larvae, male carcass, and male testes) that displayed very low pairwise correlations ($r < 0.50$) between species (Figure S4.4, Table S4.14) and genes that display adjusted p-values < 0.1 in DESeq2 tests (Table S4.15, Figures S4.5-S4.8). Raw data tables for all DESeq2 *Ae. albopictus-Ae. aegypti* tissue comparisons are found in Tables S4.16-S4.21.

When compared to *Ae. albopictus*, the *Ae. aegypti* female germline contains several upregulated genes. They include genes related to mitochondria and the production of energy (59 terms) (Table S4.15). GO terms enriched in this sample include ATP synthesis coupled proton transport, electron transfer activity, mitochondrial electron transport, and mitochondrial respiratory chain complex. In addition, several genes with DNA binding, RNA polymerase, protein binding, transcription, and translation were enriched. In *Ae. albopictus*, relatively fewer genes are

upregulated in the female germline. Genes enriched include those associated with translation, ATP binding, DNA binding, and metabolic process. In the female pupal stage, Ae. aegypti contains genes enriched for terms that include chitin binding, chitin metabolic processes, serine-type endopeptidase activity, and many uncharacterized genes. Interestingly, *Ae. albopictus* contains genes enriched for odorant and protein binding and other terms. Compared to *Ae. albopictus*, *Ae. aegypti*'s 4th larval instar program contains genes enriched for oxidoreductase activity, proteolysis, chitin binding, and steroid biosynthetic processes. In *Ae. albopictus*, upregulated genes correspond to translation, protein secretion, metabolic processes, oxidation-reduction, and metallopeptidase activity (Table S4.15). Metallopeptidases are a class of endopeptidases that are hypothesized to function in immunity and development (Vishnuvardhan et al. 2013).

In male carcasses, *Ae. aegypti* contains terms enriched for some metabolic processes, chitin binding, hydrolase activity and proteolysis. In *Ae. albopictus*, upregulated genes correspond to terms enriched for ATP binding, protein binding, and proteolysis. Based on our initial analyses of the *Ae. albopictus* samples, the male testes sample depicted major differences when compared to all stages (Figure 4.1). It was highly uncorrelated to other stages within this species and displayed a uniqueness that needed to be further investigated. When the testes sample was compared to *Ae. aegypti*, we found that more genes were upregulated in *Ae. albopictus* testes as compared to *Ae. aegypti* testes (172 versus 97 genes, respectively) (Table S4.15). The majority of these orthologous genes in *Ae. albopictus* (n = 69) are uncharacterized and their function remains unknown (Table S4.15). It would be interesting to further explore how these unknown genes affect male germline development and fertility. Several annotated genes correspond to flagellar structure including microtubules, dynein, ciliar components, and proteins associated with mitochondrial derivatives (Table S4.15). Other genes include testis-specific protein kinases, histone genes, and several genes

involved in DNA-binding transcription factor activity, presumably to regulate transcription in spermatogenesis. Several serine/threonine-protein kinases are expressed and are involved in the control of many physiological processes, like flagellar motility and muscle contraction (Cohen 1997). Cytosol aminopeptidase was also upregulated in *Ae. albopictus*. This gene is among one of the top highly expressed male-specifc genes in our *Ae. albopictus* testes analyses, with multiple copies present, and is known to be one of the most abundant sperm proteins in *Ae. aegypti* seminal fluid (Degner et al. 2019). In *Ae. aegypti* testes, we found fewer upregulated genes that include odorant binding proteins, carboxypeptidases, trypsins, and hormone-related genes (Table S4.15).

4.5 Discussion

Ae. aegypti and *Ae. albopictus* are medically important mosquitoes with the capacity to transmit a variety of human pathogens. Taking into account that these mosquitoes are vectors of dengue, Zika and chikungunya viruses, there is a potential risk of increasing the incidence of these diseases. Although *Ae. aegypti* is considered as the primary vector for these viruses, *Ae. albopictus* is emerging as another important vector (Reiter, Fontenille, and Paupy 2006). In order to contribute to the knowledge of the biological development of *Ae. albopictus*, we analyzed the whole transcriptome at different developmental stages of the life cycle. Our work will provide others with the basic foundation for their genomic studies. The observations presented here should reflect a comprehensive snapshot of the *Ae. albopictus* developmental transcriptome, an accomplishment not yet undertaken in the field-- until now. Our results provide confirmation for up to 95% (36,347/38,261) of previously annotated AALF genes.

The mosquito's life cycle can be divided into four major phases: the maternal to zygotic transition (ovary to embryos 0-8 hr); embryogenesis (from 8hr to 72 hr), diapause (0-1wk and 2-

3wks); larvae (1-4 instars) to pupae (early and late); and adults (male and female). These crucial transitions of the mosquito life cycle share many genes whose expression shows little difference between each time point. Our cluster analysis and characterization of developmental and sex-biased genes identified a number of patterns of co-regulated gene expression. We see sex-biased expression of a number of genes in the mosquito male and female germline that gives us insights into the reproductive biology of *Ae. albopictus*. Identification of loci involved in the blood meal program of the ovaries will be of interest to understand the regulation of ovarian development. In this study, we were able to depict the dynamics of genes involved in small RNA production (siRNAs, miRNAs, and piRNAs) across all development. Small RNAs in mosquitoes are known to partake in many important roles in cell development, response to stress, infection, and the silencing of transposable elements (Lucas, Myles, and Raikhel 2013). While we did not characterize small RNAs, our analysis on genes involved in small RNA production gives us insights into the roles of the small RNA pathways in *Ae. albopictus*. It would be interesting to investigate the small RNA profiles as it pertains to our results.

In our transcriptomic analysis of mosquito tissues, we discovered that the male testes showed a distinct gene expression profile that differentiated it from other tissues. Upon closer inspection, we identified 485 male-biased genes that were expressed in male testes, carcass, and male pupae. Among this list, the highest expressing genes corresponded to several uncharacterized loci, cytosol aminopeptidases, and tubulins. It would be interesting to see what functions and processes the uncharacterized genes are involved in. It is likely they may be involved in spermatogenesis, seminal fluid production, or a mating induced response. Perhaps these highly expressed genes have important roles in spermatogenesis and in the management and production of seminal fluid proteins to enable the reproductive success of *Ae. albopictus* males. The highly

divergent gene expression in the testes may suggest male-specific genes in this species are evolving rapidly (Swanson and Vacquier 2002). *Ae. albopictus* males exhibit an interesting mating strategy that involves male mosquitoes mating with multiple females in succession without regard to the availability of sperm in their testes (Oliva et al. 2013). Another strategy involves transferring male accessory gland secretions with sperm into the female to prevent further insemination. This is very similar to the mating plug seen in *Anopheline* species (Giglioli 1964). In the wild, *Ae. albopictus* mosquitoes are shown to displace *Ae. aegypti* populations in areas where they co-occur (Muzari et al. 2019). Currently, it is hypothesized that *Ae. albopictus* is able to do this because it is a superior larval competitor, however, a recent study suggested that competitive displacement is due to *Ae. albopictus* males mating with *Ae. aegypti* females resulting in female mating refractoriness (Tripet et al. 2011). This mating interference is known as 'satyrization' and has been suggested to be a form of adaptation favoring the invasive success of *Ae. albopictus* (Lounibos and Kramer 2016). In our search to understand what the highly expressed genes in male testes are, we found at least one beta-tubulin-2 gene that was duplicated. This is not surprising considering the *Ae. albopictus* genome is highly duplicated, however, it is unclear to what extent a duplication of male-biased genes can contribute to the male reproductive success of these mosquitoes. It is possible that in this species, the testes-biased genes can exhibit rapid evolution contrary to *Ae. aegypti* which experiences decelerated rates of evolution in the testes (Whittle and Extavour 2017).

The comparative *Aedes* developmental transcriptomics approach enabled us to obtain insights into the similarities and differences in developmental life stages between the two species. For example, when comparing the correlations of corresponding samples between species, the male testes has the lowest similarity and indicates an interesting difference in male germline biology in *Ae. albopictus* mosquitoes. The most similar sample/tissue between species was the 24

hr PBM ovaries. This similarity may suggest a conservation of genes involved in oogenesis at that time point. A DESeq2 analysis allowed for the exploration of our data and gave us insights into what kinds of genes were coregulated across developmental stages. Additional replicates will be needed to identify with accuracy and confidence the loci with differential expression. Overall, our results provide insight into the overall differences between these two species and list potential genes that may be involved.

In addition to providing a tool for basic molecular research on *Ae. albopictus*, the developmental transcriptome will facilitate the development of transgenesis-based control of vector populations. Regulatory elements that direct expression of transgenes in germline specific tissues will be useful for the development of gene drive mechanisms for spreading a desired trait in a mosquito population (Sieglaff et al. 2009; Akbari, Papathanos, et al. 2014). In *Ae. aegypti*, several regulatory elements able to drive gene expression in a tissue- and temporal- specific manner have been identified through extensive study (Akbari, Antoshechkin, et al. 2013) and transgenesis (Coates et al. 1999; Smith et al. 2007; Moreira et al. 2000; V. Kokoza et al. 2000). Future functional characterization of uncharacterized genes and regulatory elements may lead to the development of innovative genetic population control technologies such as precision guided sterile males (Kandul, Liu, Sanchez C, et al. 2019), and gene drive systems (Kandul, Liu, Sanchez C, et al. 2019; A. B. Buchman et al. 2018; Akbari, Chen, et al. 2014; Akbari, Matzen, et al. 2013; A. Buchman et al. 2018; Champer, Buchman, and Akbari 2016; Kandul, Liu, Buchman, et al. 2019; Li et al. 2019) which can be linked to anti-pathogen effectors (A. Buchman, Gamez, Li, Antoshechkin, Lee, et al. 2019; A. Buchman, Gamez, Li, Antoshechkin, Li, et al. 2019) potentially providing paradigm-shifting technologies to control this worldwide human disease vector. Overall, our results provide a comprehensive snapshot of gene expression dynamics in the development of

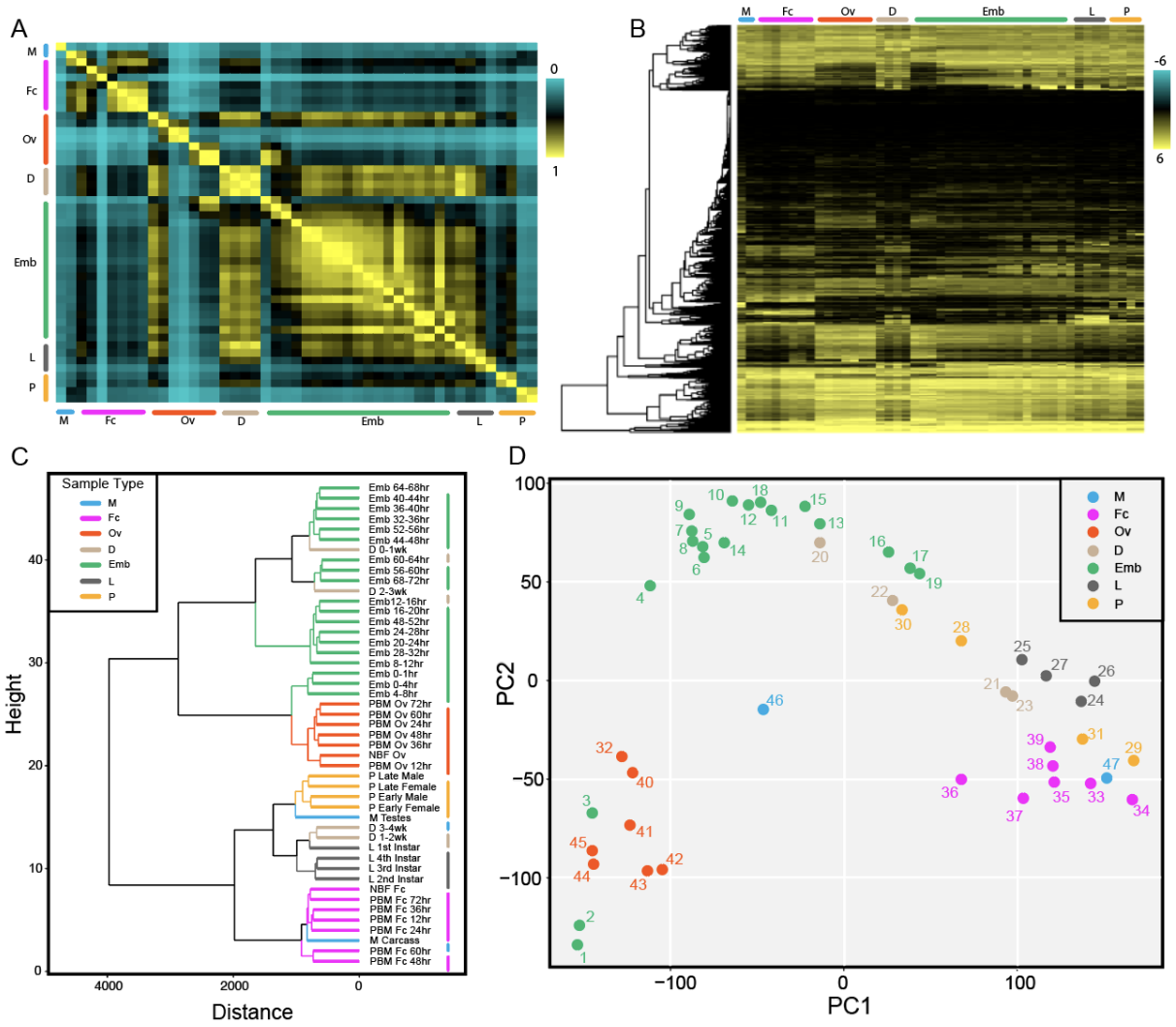
Ae. albopictus mosquitoes. The comparative analysis performed between *Ae. albopictus* and *Ae. aegypti* will be helpful in facilitating future comparative biological studies to understand the molecular basis of their differences.

4.6 Acknowledgements

Chapter 4, in full, is a reprint of the material as it appears in G3 (Genes, Genomes, Genetics). Stephanie Gamez, Igor Antoshechkin, Stelia Mendez-Sanchez, Omar S. Akbari. A comprehensive developmental transcriptome analysis of *Ae. albopictus*, an invasive mosquito. G3. (2020). The dissertation/thesis author was first author and primary investigator of this paper.

4.7 Figures

Figure 4.1 Global dynamics of gene expression. (A) Correlation matrix of all RNA seq timepoints for all known *Ae. albopictus* genes. (B) Hierarchical clustering heat map of albopictus genes across all developmental stages. FPKM values were log2(x+1) transformed and were scaled to plot the z-scores. (C) Dendrogram of *Ae. albopictus* samples clustering similar life stages closer together. Plot depicts the close relationship between all developmental samples. (D) PCA clustering of *Ae. albopictus* samples depicts clustering of life stages who show close similarity. PCA plot is in agreement with clustering dendrogram. Each point is labeled with the "Order" number they are assigned to from Table S1. For A-D, the second testes replicate was not shown. For A-D, the major developmental groups are indicated by color bars and are organized as follows: M (blue, male testes, male carcass), Fc (pink, NBF carcass, and multiple timepoints PBM: 12hr, 24hr, 36hr, 48hr, 60hr, and 72hr), Ov (orange, NBF ovaries, and multiple ovarian timepoints PBM: 12hr, 24hr, 36hr, 48hr, 60hr, and 72hr), D (tan, diapause at multiple timepoints: 0-1wk, 1-2wk, 2-3wk, and 3-4wk), Emb (embryo at multiple timepoints: 0-1 hr, 0-2 hr, 2-4 hr, 4-8 hr, 8-12 hr, 12-16 hr, 16-20 hr, 20-24 hr, 24-28 hr, 28-32 hr, 32-36 hr, 36-40 hr, 40-44 hr, 44-48 hr, 48-52 hr, 52-56 hr, 56-60 hr, 60-64 hr, 64-68 hr, and 68-72 hr embryos), L (gray, larvae 1st, 2nd, 3rd, and 4th instar larvae stages), and P (yellow, pupae, early male and female, and late male and female pupae stages).


A
M
Fc
Ov
D
Emb
L
P
0
1
B
-6
6
C
Sample Type
Height
Distance
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2000
0
40
30
20
10
Emb 64-68hr
Emb 40-44hr
Emb 36-40hr
Emb 32-36hr
Emb 52-56hr
Emb 44-48hr
D 0-1wk
Emb 60-64hr
Emb 56-60hr
Emb 68-72hr
D 2-3wk
Emb12-16hr
Emb 16-20hr
Emb 48-52hr
Emb 24-28hr
Emb 20-24hr
Emb 28-32hr
Emb 8-12hr
Emb 0-1hr
Emb 0-4hr
Emb 4-8hr
PBM Ov 72hr
PBM Ov 60hr
PBM Ov 24hr
PBM Ov 48hr
PBM Ov 36hr
NBF Ov
PBM Ov 12hr
P Late Male
P Late Female
P Early Male
P Early Female
M Testes
D 3-4wk
D 1-2wk
L 1st Instar
L 4th Instar
L 3rd Instar
L 2nd Instar
NBF Fc
PBM Fc 72hr
PBM Fc 36hr
PBM Fc 12hr
PBM Fc 24hr
M Carcass
PBM Fc 60hr
PBM Fc 48hr
D
PC2
PC1
100
50
0
−50
−100
−100
0
100

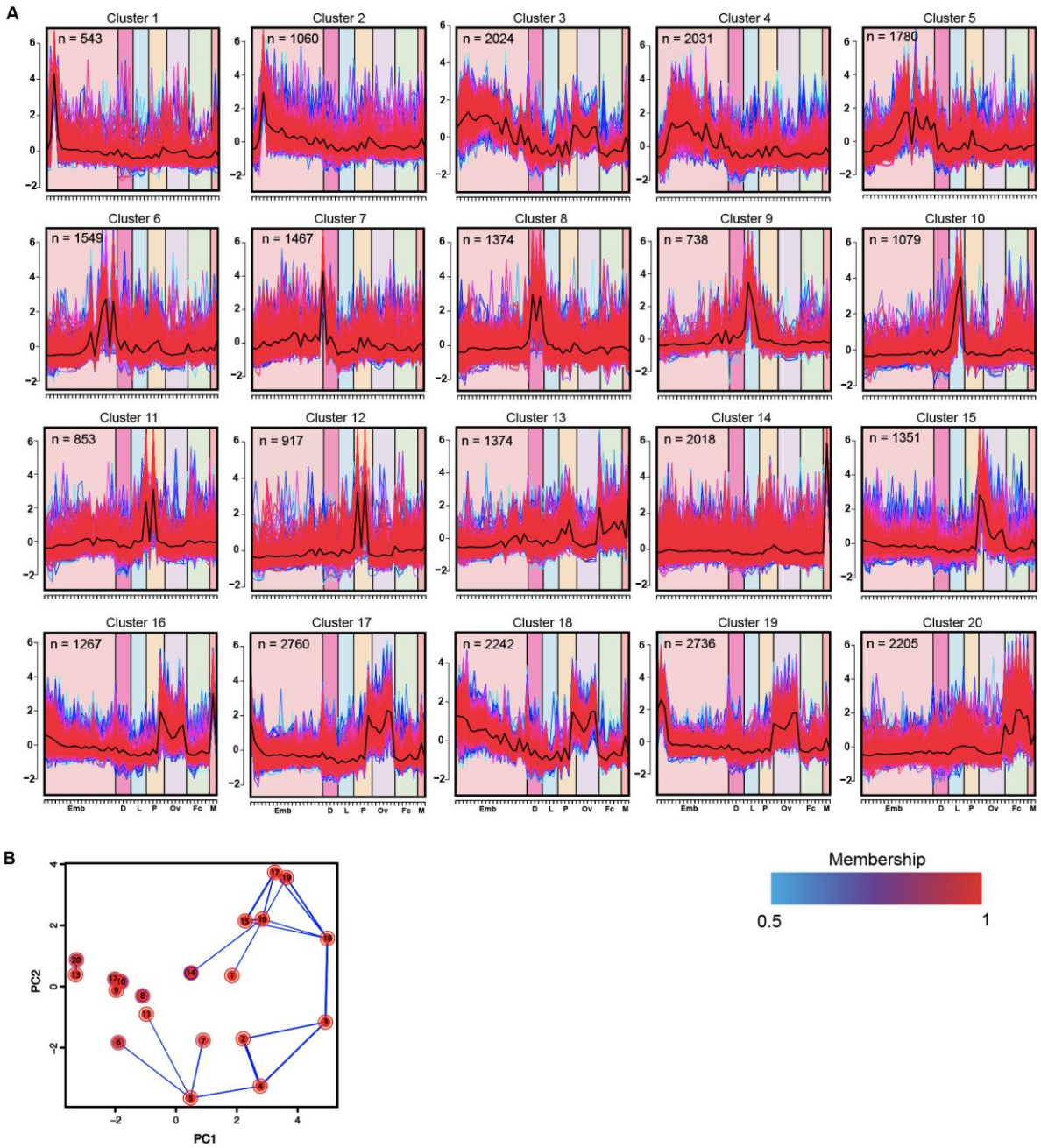


Figure 4.2 Soft clustering and principal component analysis on *Ae. albopictus* genes. (A) Twenty albopictus gene expression profile clusters were identified through soft clustering. Each gene is assigned a line color corresponding to its membership value, with red (1) indicating high association. The developmental groups are indicated by symbols on the x-axis. (B) Principal component analysis shows relationships between the 20 clusters, with thickness of the blue lines between any two clusters reflecting the fraction of genes that are shared. N, the number of genes in each cluster.

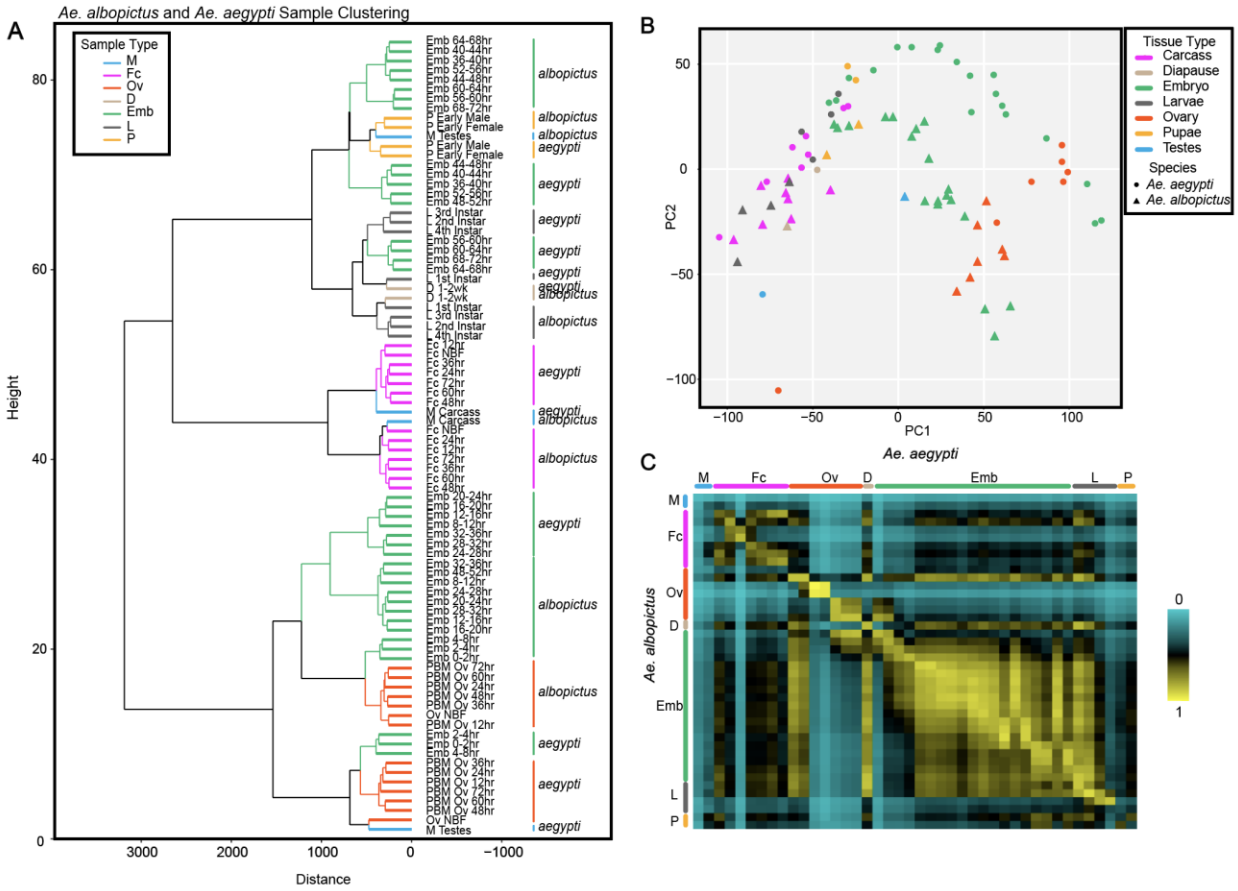


Figure 4.3 Orthology analysis of *Ae. aegypti* and *Ae. albopictus* samples across corresponding developmental time points. Orthologs were identified by best reciprocal BLAST hit approach and expression values of orthologous genes were determined by aligning RNA-seq reads from the *Ae. aegypti* and *Ae. albopictus* samples to corresponding genomes using STAR (e.g. *Ae. aegypti* reads were aligned to *Ae. aegypti* genome) and quantifying them with featureCounts (Materials and Methods). Species-specific expression values of orthologous genes were used for clustering and PCA analyses. (A) Dendrogram and (B) principal component analysis (PCA) on similar life stage sample types in both species. Both clustering analyses agree with each other indicating the close relationships of similar genes among each developmental time point between species. Interestingly, the *Ae. albopictus* male testes sample clusters distantly from *Ae. aegypti* testes which may indicate a significant difference between the two species. (C) Heat map of calculated Pearson correlations on samples between species. *Ae. albopictus* samples are represented on the vertical axis of the heat map while *Ae. aegypti* samples are represented on the horizontal axis. Life stages are indicated by the similar colored bars for both species.

4.8 Supplemental Figures

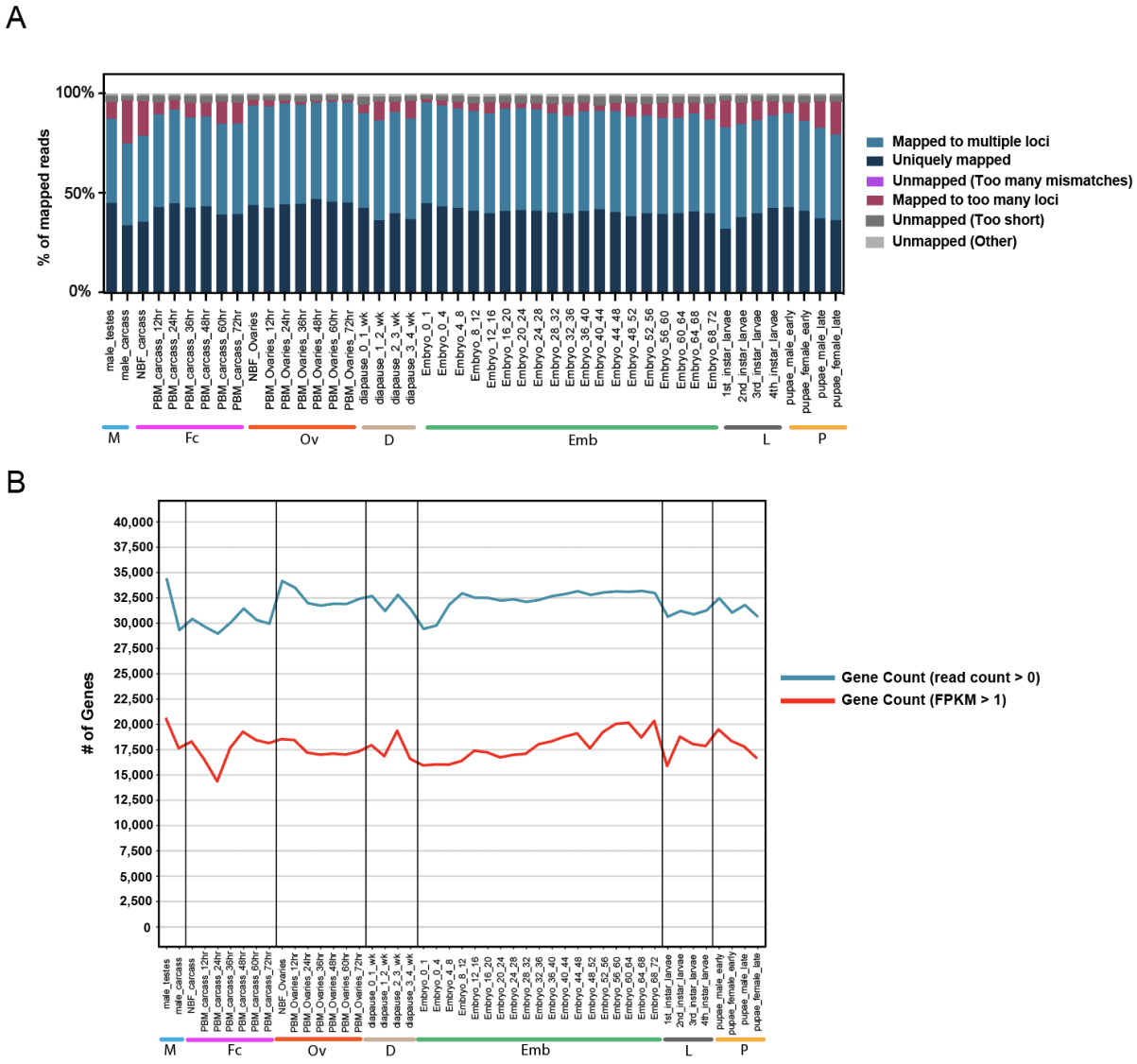


Figure S4.1 Read mapping statistics and read count data across all developmental stages. (A) Read mapping analysis of *Ae. albopictus* developmental samples. The distribution reflects percentage of reads mapped to multiple loci (blue), uniquely mapped fragments (dark blue), unmapped fragments with too many mismatches (purple-pink), fragments mapped to too many loci (maroon), unmapped fragments that are too short (dark gray) or other (gray). (B) The number of genes expressed at FPKM > 1 (red), and with non-zero count values (blue) were plotted across all developmental timepoints. Mapping statistics can be found in Supplemental Table S4.3.

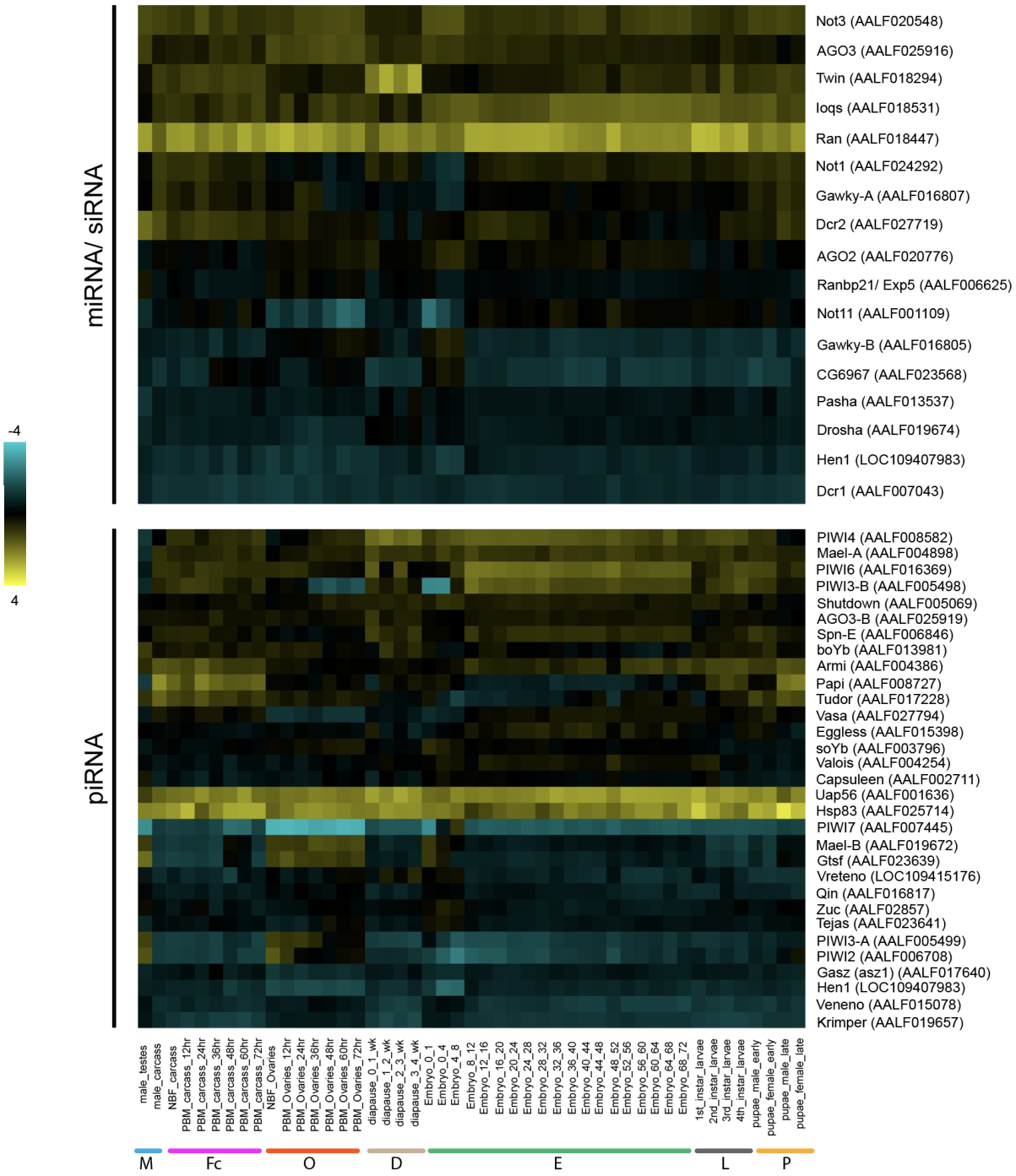


Figure S4.2 Expression dynamics of protein genes involved in miRNA, siRNA and piRNA pathways. Heat map depicts the z-scores of log2(FPKM + 1) transformed FPKM values. Gene names are listed on the right hand side of the map with their corresponding *Ae. albopictus* AALF IDs in parentheses. Developmental stages are on the bottom of the maps.

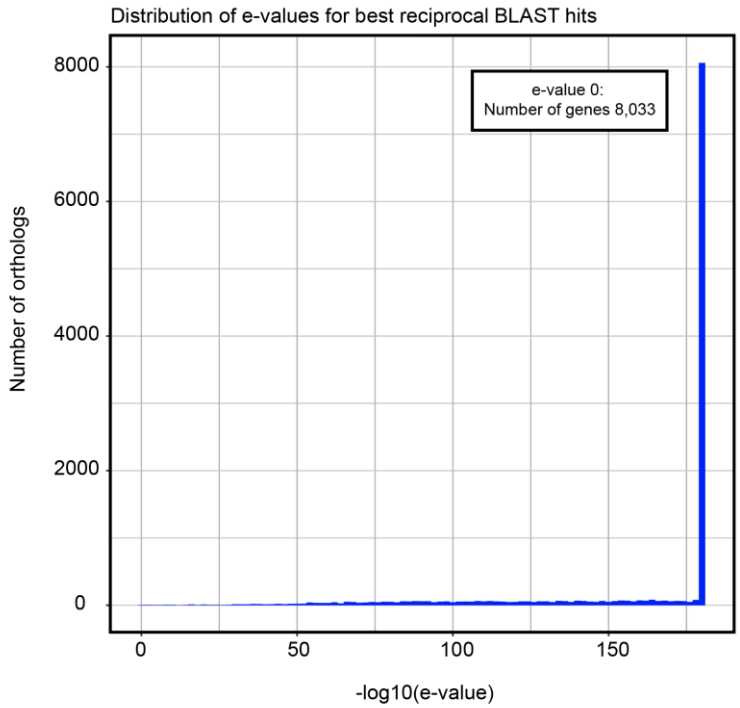


Figure S4.3 E-value score distribution for best reciprocal BLAST hits for *Ae. aegypt*i and *Ae. albopictus* orthologs. For the purpose of plotting -log10(e-value), e-value of 0 was set to 1e-181. Out of 11,687 identified ortholog pairs, 8,033 resulted e-values of 0.

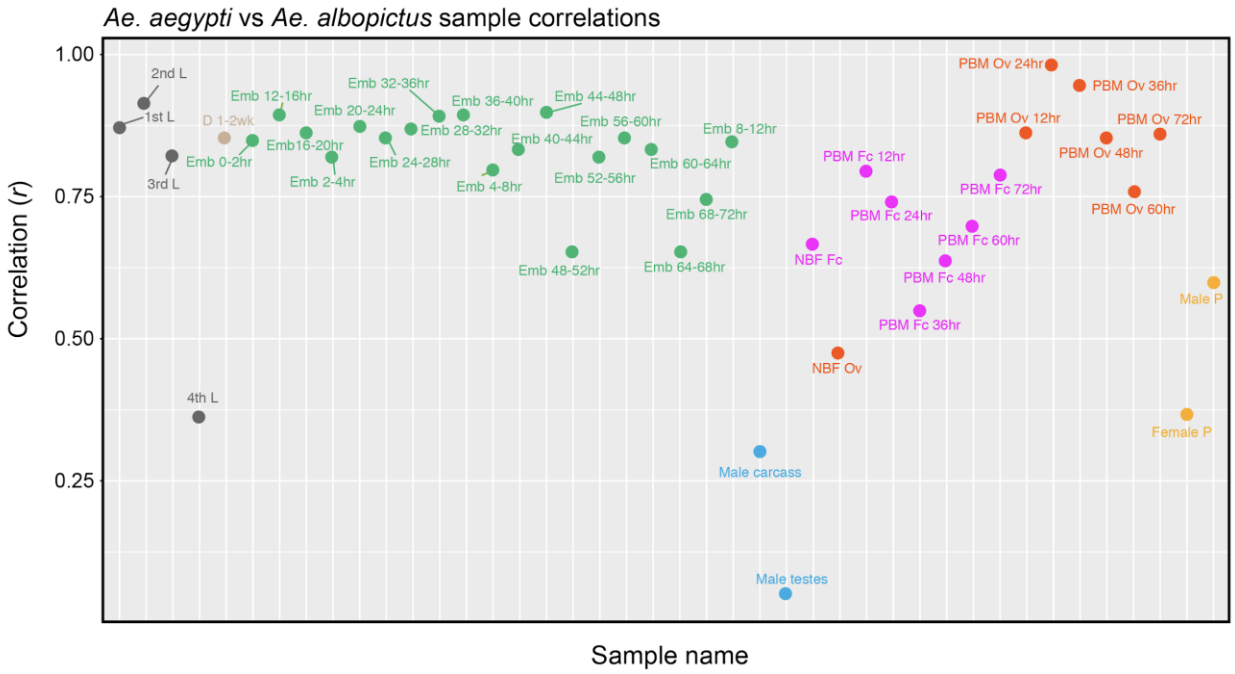


Figure S4.4 Sample correlations of each developmental time point between *Ae. aegypti* and *Ae. albopictus*. Correlation values were determined by comparing the gene expression of orthologs between the species. The male testes, male carcass, 4th larval instar, and female pupae are highly dissimilar between the two species. The major developmental groups are indicated by color and are organized as follows: M (blue, male testes, male carcass), Fc (pink, NBF carcass, and multiple timepoints PBM: 12hr, 24hr, 36hr, 48hr, 60hr, and 72hr), Ov (orange, NBF ovaries, and multiple ovarian timepoints PBM: 12hr, 24hr, 36hr, 48hr, 60hr, and 72hr), D (tan, diapause at multiple timepoints: 0-1wk, 1-2wk, 2-3wk, and 3-4wk), Emb (embryo at multiple timepoints: 0-1hr, 0-2 hr, 2-4 hr, 4-8 hr, 8-12 hr, 12-16 hr, 16-20 hr, 20-24 hr, 24-28 hr, 28-32 hr, 32-36 hr, 36-40 hr, 40-44 hr, 44-48 hr, 48-52 hr, 52-56 hr, 56-60 hr, 60-64 hr, 64-68 hr, and 68-72 hr embryos), L (gray, larvae 1st, 2nd, 3rd, and 4th instar larvae stages), and P (yellow, pupae, early male and female, and late male and female pupae stages). Correlation values for each developmental stage are listed in Supplementary Table S4.14.

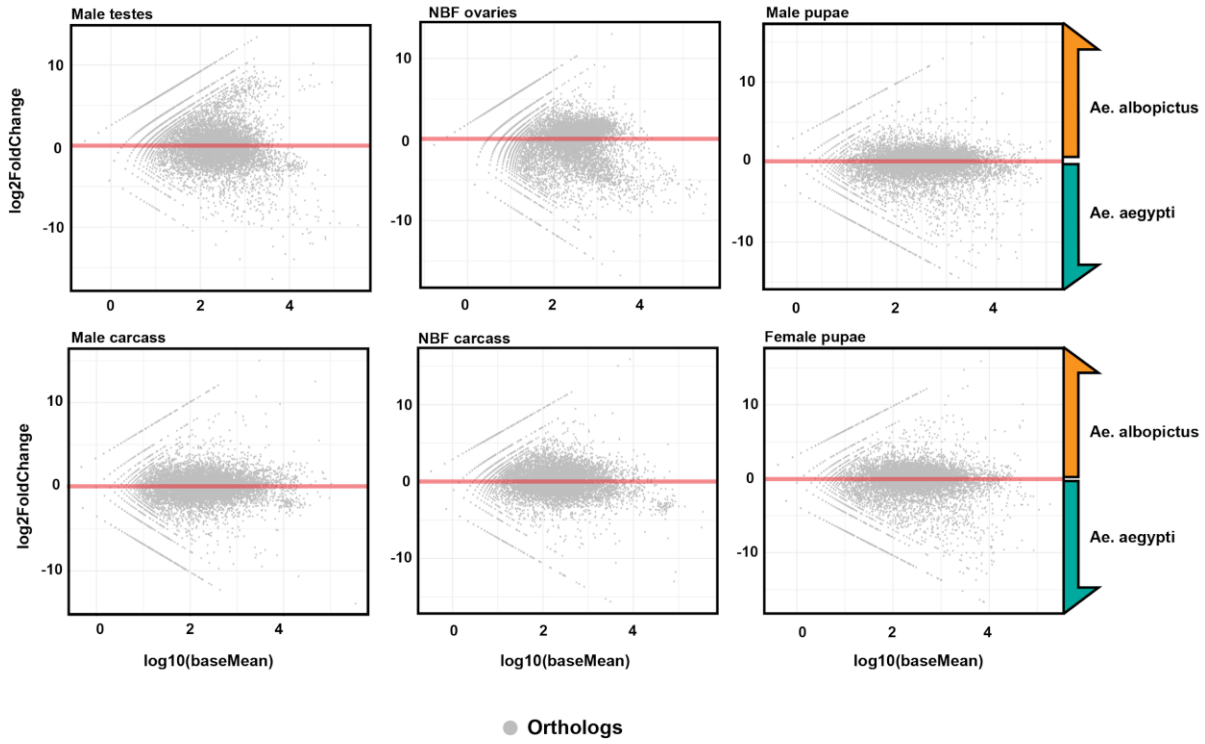


Figure S4.5 MA plots depicting expression of orthologous genes in sex specific tissues. Sex-specific tissues analysed were male testes, male carcass, NBF ovaries, NBF female carcass, male and female pupae. The y-axis represents the log2FoldChange and the x-axis represents the log10(baseMean). The red line represents the center of the plot (where log2FoldChange equals 0). The top of the plots indicate genes that are highly expressed in *Ae. albopictus* while genes found below the 0 transparent red line correspond to *Ae. aegypti.* Significance is excluded from plots due to our one replicate analysis. Data can be found in Supplemental Table S4.15.

Figure S4.6 MA plots depicting expression of orthologous genes in blood-fed female ovaries and carcasses at different 12-hr time points. The y-axis represents the log2FoldChange and the x-axis represents the log10(baseMean). The red line represents the center of the plot (where log2FoldChange equals 0). The top of the plots indicate genes that are upregulated in *Ae. albopictus* while genes found below the 0 transparent red line correspond to *Ae. aegypti*. Significance is excluded from plots due to our one replicate analysis. Data can be found in Supplemental Table S4.15.

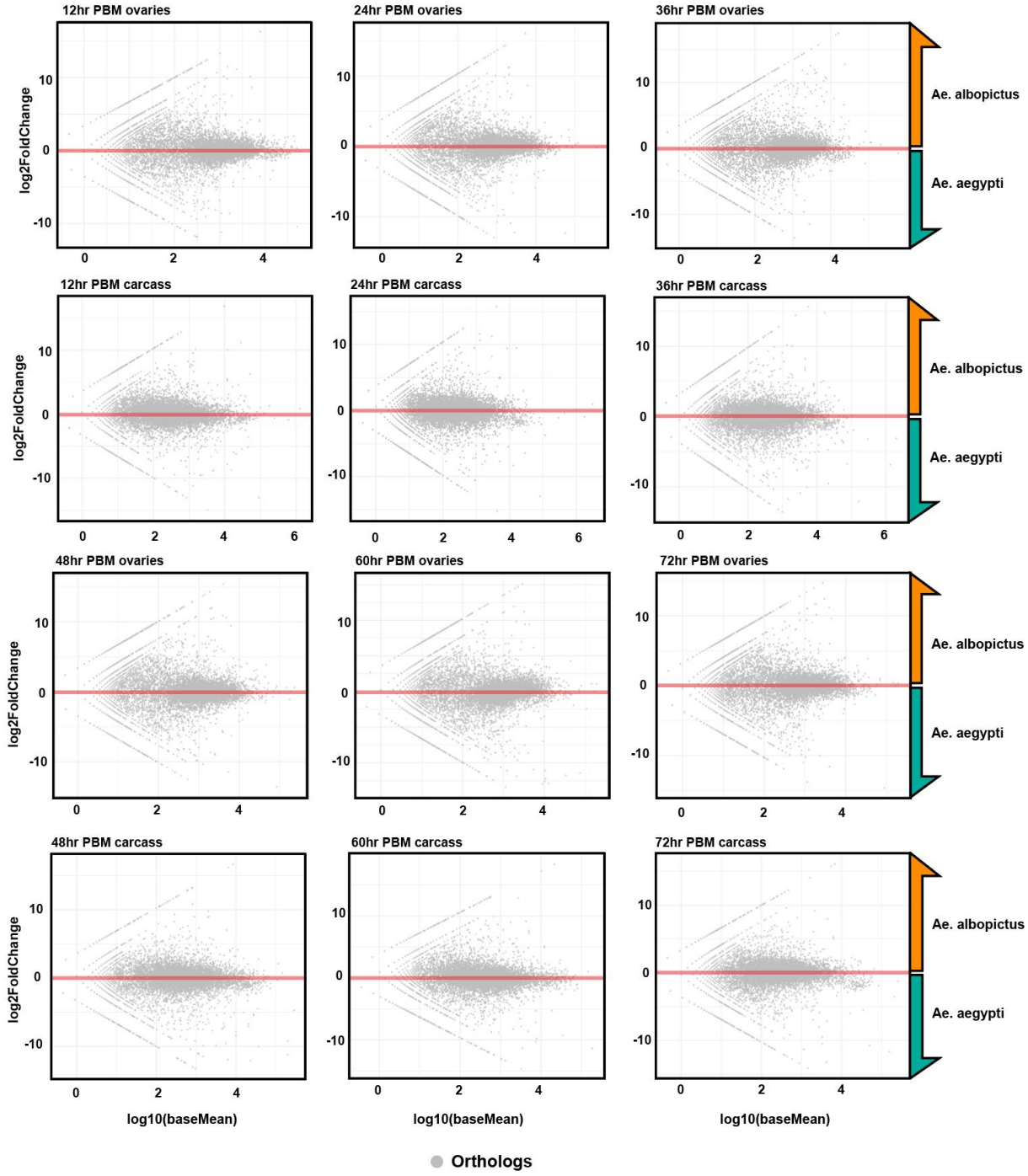
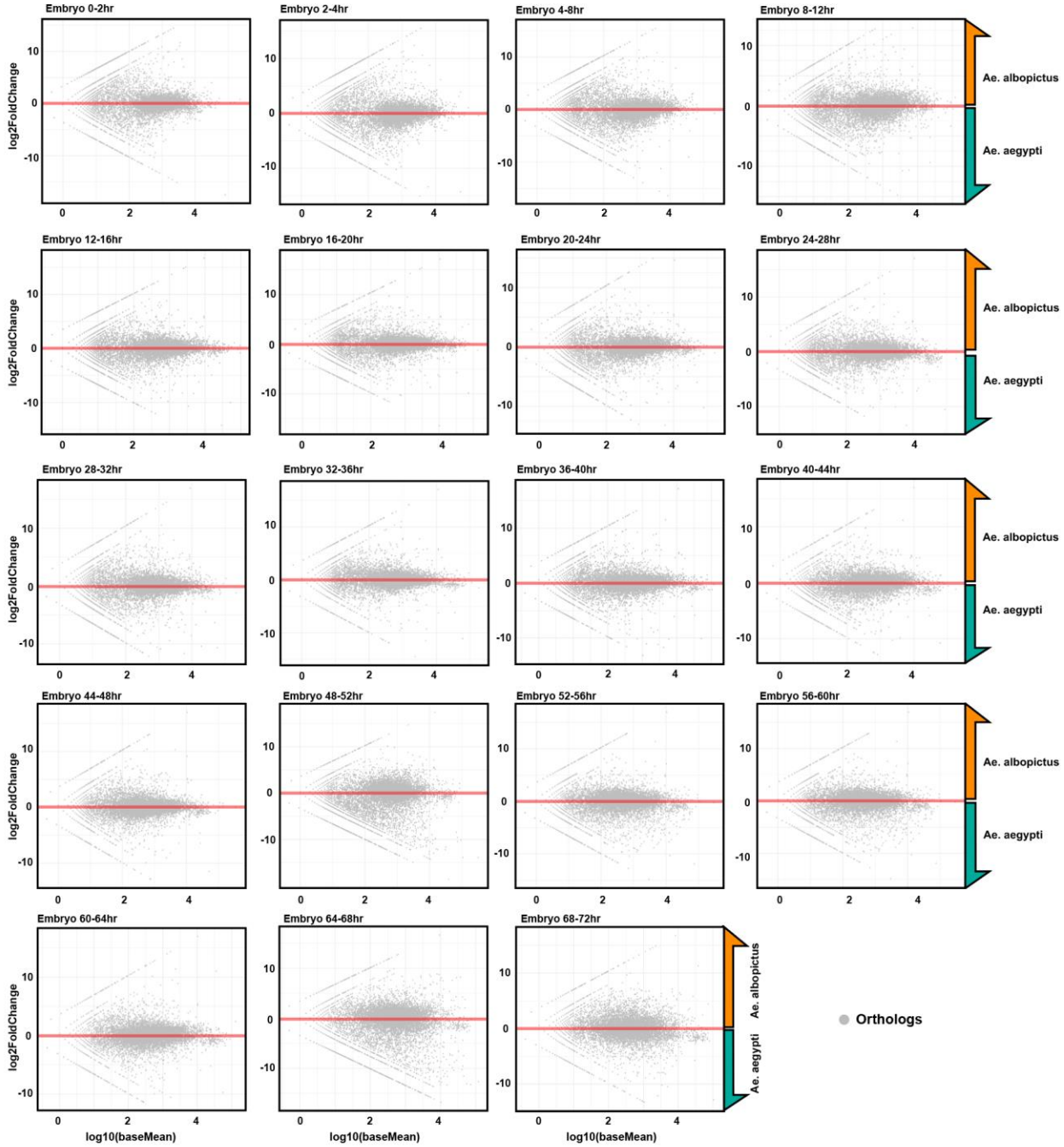

12hr PBM ovaries
24hr PBM ovaries
36hr PBM ovaries
12hr PBM carcass
24hr PBM carcass
36hr PBM carcass
48hr PBM ovaries
60hr PBM ovaries
72hr PBM ovaries
48hr PBM carcass
60hr PBM carcass
72hr PBM carcass
log2FoldChange
log10(baseMean)
Ae. albopictus
Ae. aegypti
Orthologs

Figure S4.7 MA plots depicting expression of orthologous genes during embryogenesis of *Ae. albopictus* compared to *Ae. aegypti*. The y-axis represents the log2FoldChange and the x-axis represents the log10(baseMean). The red line represents the center of the plot (where log2FoldChange equals 0). The top of the plots indicate genes that are highly expressed in *Ae. albopictus* while genes found below the 0 transparent red line correspond to *Ae. aegypti.* Data can be found in Supplemental Table S4.15.


Embryo 0-2hr
Embryo 2-4hr
Embryo 4-8hr
Embryo 8-12hr
Embryo 12-16hr
Embryo 16-20hr
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Embryo 24-28hr
Embryo 28-32hr
Embryo 32-36hr
Embryo 36-40hr
Embryo 40-44hr
Embryo 44-48hr
Embryo 48-52hr
Embryo 52-56hr
Embryo 56-60hr
Embryo 60-64hr
Embryo 64-68hr
Embryo 68-72hr
log2FoldChange
log10(baseMean)
Ae. albopictus
Ae. aegypti
Orthologs

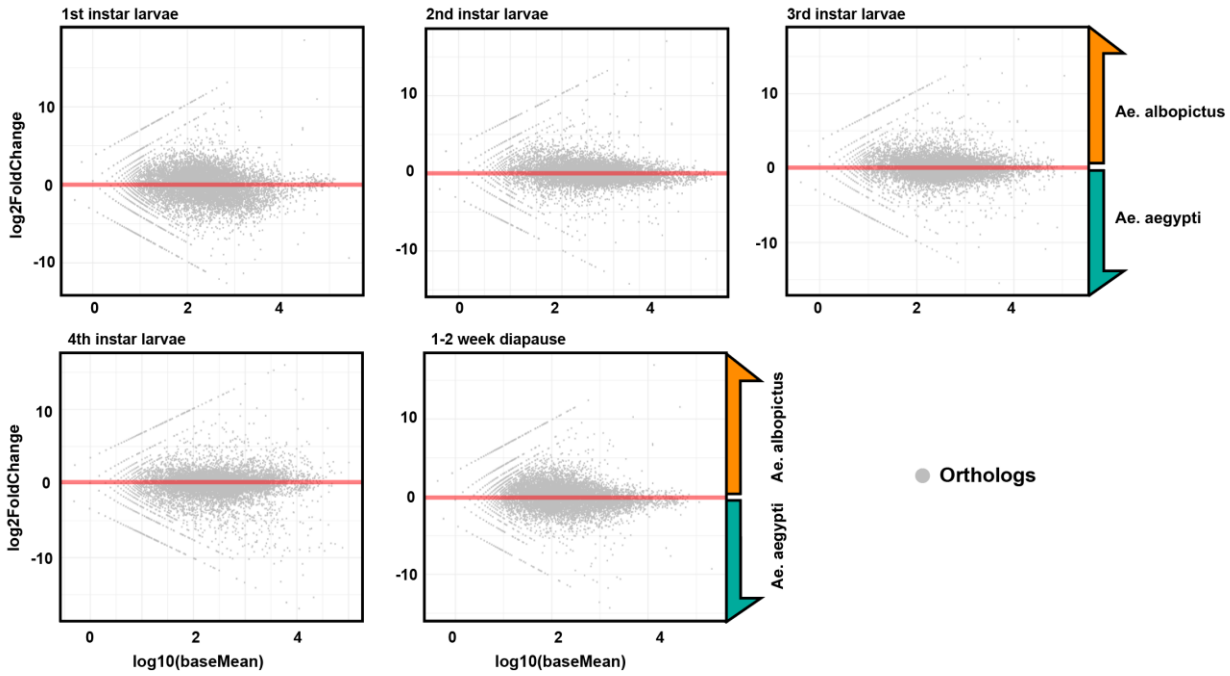

Figure S4.8 MA plots depicting expression of orthologous genes in the larval instars and diapause samples of *Ae. albopictus* compared to *Ae. aegypti*. The y-axis represents the log2FoldChange and the x-axis represents the log10(baseMean). The red line represents the center of the plot (where log2FoldChange equals 0). The top of the plots indicate genes that are highly expressed in *Ae. albopictus* while genes found below the 0 transparent red line correspond to *Ae. aegypti.* Data can be found in Supplemental Table S4.15.

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Chapter 5: Development of alternative transactivational binary expression systems in *Drosophila melanogaster*

5.1 Abstract

Controlling gene expression is an instrumental tool for biotechnology, as it enables the dissection of gene function, affording precise spatial-temporal resolution. To generate this control, binary transactivational systems generally employ a modular activator consisting of a DNA binding domain fused to activation domain(s) used to increase target gene expression. For fly genetics, many binary transactivational systems have been exploited in vivo; however as the study of complex problems often requires multiple systems that can be used in parallel, there is a need to identify additional bipartite genetic systems. To expand the toolbox, we tested multiple bacterially-derived binary transactivational systems in *Drosophila melanogaster*. Our work provides the first characterization of these systems in an animal model. For each system we demonstrate robust tissue-specific spatial transactivation of reporter gene expression, enabling future studies to exploit these transactivational systems for molecular genetic studies.

5.2 Introduction

Precise regulation of gene expression is instrumental in biological applications such as therapeutics (Kemmer et al. 2010) and pharmaceuticals (Sharpless and Depinho 2006), where long-term regulation of gene expression for gene therapy is crucial following rational cell reprogramming in tissue engineering (Fussenegger et al. 1998) or is required to build sensors for synthetic gene circuits (Deans, Cantor, and Collins 2007; Kramer and Fussenegger 2005). This precise control is currently afforded by synthetic binary expression systems, which are engineered

transgenes that respond to the presence of modified proteins and chemical molecules (ligands). More specifically, these systems generally couple a synthetic transcription factor with a transactivation domain that binds to specific operator sites (Triezenberg, Kingsbury, and McKnight 1988). These systems can control gene expression in a temporal- and tissue-specific manner, using appropriate regulatory elements to express transactivators. This controlled expression is especially important for toxic gene products or temporal/tissue-specific knock-down of an essential gene, which would be otherwise impossible due to their deleterious effect on the organism.

While several binary transactivation systems exist, only a handful have been shown to function in vivo in *Drosophila melanogaster* (reviewed (Venken, Simpson, and Bellen 2011)), therefore we sought to further expand this powerful molecular genetic toolbox. For example in flies, transactivation systems have been used extensively in vivo affording spatial control including: Gal4-UAS adapted from yeast (Brand and Perrimon 1993), the Q-system adapted from the bread mold Neurospora crassa (Potter et al. 2010), and several systems derived from bacteria including the LexA/LexAop (Lai and Lee 2006), the Tet system using tTA/TRE (Bello, Resendez-Perez, and Gehring 1998), transcription activator-like effectors (TALEs)(Toegel et al. 2017), and recently even CRISPR/dCas9-VPR based transactivators (Lin et al. 2015; Jia et al. 2018). In addition to spatial control, some of these systems also afford temporal control by exploiting small-molecule triggers to fine-tune expression in a dose-dependent manner. For example, the Gal4-UAS system utilizes mifepristone (RU486) (Nicholson et al. 2008; Robles-Murguia et al. 2019), and temperature by using a temperature sensitive allele of GAL80, GAL80ts (McGuire et al. 2003), or trimethoprim by incorporating a destabilizing domain (Sethi and Wang 2017). Other chemically-controlled systems include the Tet system which uses tetracycline/doxycyline (Bello, Resendez-

Perez, and Gehring 1998; Bieschke, Wheeler, and Tower 1998), and the Q-system which uses quinic acid (Potter et al. 2010).

Despite this desirable level of precise spatial-temporal control, concerns have been raised over their potential side-effects in animals. For example, due to the negative fitness effects of RU486 at certain concentrations, the Gal4-UAS system may not be ideal (Landis et al. 2015; Yamada et al. 2017). Moreover, the use of temperature in flies can have a significant impact on the behavior and physiology (Parisky et al. 2016). Tetracycline/doxycycline has also been reported to have negative physiological impacts (Chatzispyrou et al. 2015; Moullan et al. 2015), including imparied mitochondrial function (Zeh et al. 2012), which may affect experimental outcomes. While the Q-system has been demonstrated to be efficient and has no documented side effects using quinic acid, this system requires both an additional genetic component, termed QS, to suppress gene expression and the supplementation of quinic acid for de-repression of QS protein (Potter et al. 2010).

Herein, we sought to characterize additional binary systems to expand the *Drosophila* genetic tool box. We tested four bacterially derived systems by encoding them in Drosophila strains including, p-CymR operon from *Pseudomonas putida* (Mullick et al. 2006), PipR operon from *Streptomyces coelicolor* (Fussenegger et al. 2000), TtgR operon from *Pseudomonas putida* (Gitzinger et al. 2009), and the VanR operon from *Caulobacter crescentus* (Gitzinger et al. 2012). To characterize these systems, we exploited a novel dual-luciferase reporter system incorporating GFP enabling both quantification and visualization of gene-expression levels, respectively, as compared to the widely used Tet-OFF system (tTA). Additionally, we tested the systems' ability to be controlled via small molecules, which may provide avenues for further optimization. Overall, our results demonstrate the robust spatial transactivational potential of these control reporter

systems and validate their relevance for future studies. This work is the first report of these particular prokaryotic systems engineered in *Drosophila* and provides the field with additional spatially-controlled transactivational systems that can be used as genetic circuits.

5.3 Methods

5.3.1 Plasmid construction

For the construction of driver transgenes, the following cloning strategies were first performed: To generate the vanTA driver (vector 907F1), an attB cutter backbone containing a multiple cloning site was digested using restriction enzymes SwaI and XbaI. The following PCR products were inserted via Gibson cloning: a Flightin (Fln) promoter fragment amplified from *Drosophila* genomic DNA using primers 907F1.F1 and 907F1.R1, a vanR repressor protein from a gene-synthesized plasmid using primers 907F1.F2 and 907F1.R2, and finally, a VP16-SV40 fragment amplified from a gene-synthesized plasmid using primers 907F1.F3 and 907F1.R3. To generate the cymTA driver (vector 907H1), an attB cutter backbone containing a multiple cloning site was digested using restriction enzymes SwaI and XbaI. The following PCR products were inserted via Gibson cloning: a Flightin (Fln) promoter fragment amplified from *Drosophila* genomic DNA using primers 907F1.F1 and 907H1.R1, a cymR repressor protein from a gene-synthesized plasmid using primers 907H1.F2 and 907H1.R2, and finally, a VP16-SV40 fragment amplified from a gene-synthesized plasmid using primers 907H1.F3 and 907F1.R3.

In a separate cloning strategy, we cloned in a longer variant of the VP16 domain (VP16') in the ttgTA and pipTA systems to see if this VP16' would also provide robust activation of reporter genes in our system. We used vector 907F1 as a backbone to save two PCR amplification steps (Fln promoter fragment and the SV40 fragment). To generate the pipTA driver (vector

907K), we digested plasmid 907H1 with AscI and BglII restriction enzymes. The following PCR products were inserted via Gibson cloning: the pipR repressor sequence amplified from a gene-synthesized plasmid using primers 907K.F2 and 907K.R2, and the VP16 sequence amplified from a gene-synthesized vector with primers 907K.F3 and 907K.R3. To generate the ttgTA driver (vector 907L), we digested plasmid 907H1 with AscI and BglII restriction enzymes. The following PCR products were inserted via Gibson cloning: the ttgR sequence amplified from a gene synthesized plasmid using primers 907L.F2 and 907L.R2, and the VP16 sequence which was amplified from a gene synthesized vector with primers 907L.F3 and 907L.R3. We chose the Tet-OFF system as the positive control to compare novel systems to a widely used repressible system. For this, the TetR-VP16 sequence was amplified from an Oxitec plasmid OX1124 (Morrison et al. 2012) using primers 1025.c1 and 1025.c2 and cloned into a AscI/BglII digested 907F1 vector. All primers used for driver constructs are listed in Table S5.1.

To engineer responder transgenes, several cloning steps were performed. First an attB cutter vector was digested with AscI and XbaI. The following were added via Gibson cloning to create intermediate vector 908-1a: firefly luciferase, amplified from a gene synthesized vector using primers Firefly.F and Firefly.R and a T2A-GFP-p10-3'UTR sequence amplified from a previously described vector (Kandul et al. 2019) using primers GFP.F and GFP.R. Then, 908-1a was digested with XhoI, and the following components were added via Gibson cloning to generate intermediate vector 908-1b: an Hsp70 minimal promoter, amplified synthetically using primers Hsp70.F and Hsp70.R and a UASp promoter (Rørth 1998) amplified from the pWALIUM22 plasmid using primers UAS.F and UAS.R. Finally, 908-1b was digested with XbaI to add an SV40-Renilla-luciferase-ubiquitin sequence that was amplified from a previously engineered vector 1052 (unpublished) with primers UbiqRen.F and UbiqRen.R to make vector 908-1c. Then, OA1c was

digested with XhoI/PacI to insert the operator sequences for each system upstream of the minimal Hsp70 promoter. Specifically, the operator sequences (ttgO) for the ttgTA system were amplified from a gene-synthesized vector using primers ttgO.F and ttgO.R to make the final vector 908E. For the pipTA system, operator sequences (pipO) were amplified synthetically using primers 908A17 and 908A18 to make the final vector 908G. For the tTA system, operator sequences (tetO) were synthetically amplified using primers 908A11 and 908A12 to make the final vector 908H. For the vanTA system, the operator sequences (vanO) were amplified using primers 908A13 and 908A14 to make the final vector 908I. For the cymTA system, operator sequences (cymO) were amplified using primers 908A15 and 908A16 to make the final vector 908J. All primers used for responder constructs are listed in Table S5.2. For a complete list of vectors, Addgene ID numbers, and vector descriptions, please refer to Table S5.3. Plasmid DNA and complete annotated DNA plasmid sequences maps can be found at www.Addgene.com.

5.3.2 Fly rearing and genetic crosses

Rainbow Transgenics (Camarillo, CA, USA) performed all of the fly injections. All driver and responder constructs were injected into a transgenic 3rd chromosome attP site line marked with 3xP3-RFP (Bloomington Drosophila Stock Center (BSC), Bloomington, IN, USA; RRID: BDSC_24486, y[1] M{RFP[3xP3.PB] GFP[E.3xP3]=vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-86Fa). Recovered transgenic lines containing the construct at the 3rd chromosome site were balanced on the 3rd chromosome using a double-chromosome balancer line (w1118; CyO/Sp; Dr/TM6C, Sb1). Single homozygous transgenic driver and responder flies were maintained as separate lines. Flies were maintained on cornmeal, molasses, and yeast medium (Old Bloomington Molasses Recipe) at 25°C with a 12H/12H light/dark cycle. To assess system

activation, we used Instant Drosophila Food (Formula 4–24) from the Carolina Biological Supply Company. In each fly vial (FlyStaff.com), 1.1 g of dry food was mixed with 5 ml of distilled water. To obtain transheterozygous flies, driver strains were crossed to the responder strains in treated or non-treated food. As a control, the responder strain was crossed to a WT (w[1118]) strain to produce heterozygous responders. To assess system suppression with ligand, instant food was supplemented with doxycycline, cumate, phloretin, vanillic acid, or virginiamycin M1 in varying concentrations using the compound solutions described below. All driver and responder strains were deposited to the Bloomington Drosophila Stock Center, and their corresponding BDSC IDs are listed in Table S5.3.

5.3.3 Compound solutions

Doxycycline (195044, MP Biomedicals LLC), with a half-life of 11–12 hrs (Graham and Pile 2016), was prepared as a stock solution of 1,000 µg/mL in 100% ethanol. To make concentrations of 1 µg/mL and 10 µg/mL for 1x and 10x treatments, respectively, stock solution was diluted in distilled H2O. Cumate (QM100A-1, SBI) arrived as a 300 mg/mL (10,000X) stock solution in 500 µL. To make 1x and 10x treatment solutions, two serial dilutions at 1:10 were first performed with distilled H2O to reach a workable concentration of 300 µg/mL. Then 2.5 µg/mL (low treatment) and 25 µg/mL (high treatment) working concentrations were generated in distilled H2O. Phloretin (P7912, Sigma-Aldrich), with a half-life of 70 hrs (Gitzinger et al. 2009), was prepared as a stock solution of 10 mg/mL in 100% ethanol. The phloretin stock solution was diluted in distilled H2O to 4 µg/mL and 40 µg/mL for the low and high ligand treatments, respectively. Vanillic acid (H36001, Sigma-Aldrich), with a 7 min half-life (Yrbas et al. 2015), was prepared as a stock solution at 1,680 µg/mL by dissolving the powder in distilled H2O over a

hot magnetic spin plate. Since vanillic acid is an acidic compound, 3 M of NaOH was added to neutralize the solution to a pH of 7.0 for fly food. Working solutions were made by diluting the stock solution in distilled H2O to a final concentration of 10 µg/mL and 100 µg/mL for the low and high ligand treatments, respectively. Virginiamycin M1 (V2753, Sigma-Aldrich), with a half-life of 4–5 hrs (Gitzinger et al. 2009; Kwon 2017), was prepared as a stock solution at 500 µg/mL in 100% ethanol. Working concentrations of 0.5 µg/mL and 5 µg/mL for the low and high ligand treatments respectively, were made by diluting the stock solution in water. Fly food treatments were set up by adding 1.1g of Formula 2-24 Instant Drosophila Medium (#173218, Carolina) to an empty fly vial and adding 5mL of working solution. The solution was allowed to sit for at least 4 hours before adding flies.

5.3.4 Imaging

Flies were scored and imaged on the Leica M165FC fluorescent stereomicroscope equipped with the Leica DMC2900 camera. Images were done under constant conditions.

5.3.5 Luciferase assays

The Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) was used to measure firefly luciferase expression in response to transactivation or repression. To measure luciferase consistently, 3-day old male flies were collected in a 1.5 µL microcentrifuge tube and stored in the -80C before lysing. Treatments were done in triplicate. Each replicate contained five sub-replicates with five flies each. To lyse the sample, the Passive Lysis Buffer 5X was diluted in distilled H_2O at 1:5 to make a 1x lysis buffer. Then, 40 µL of lysis buffer was used to mechanically disintegrate the sample with a plastic pestle and an additional 40 µL of buffer was used to wash

the remaining tissue off the pestle into the tube. To remove the tissue, lysed samples were subjected to a 15 min centrifuge spin at 10,000 rpm. 75 µL of the supernatant (without tissue) was removed and placed into a clean tube and stored in the -80C. Before measuring luciferase activity, Luciferase Assay Reagent II (LARII) and Stop and Glo® reagents were prepared beforehand according to the manufacturer's instructions. Firefly luciferase activity was first measured by adding 100 µL of LARII in a tube containing 5 µL of lysed sample and placed in GloMax® 20/20 Luminometer (Promega, Madison, WI) to measure luminescence in relative luciferase units (RLU) for an integration time of 10 s. Then 100 µL Stop and Glo® was added to measure Renilla luciferase for 10s. Each measurement was recorded in an excel spreadsheet and later organized for calculations.

5.3.6 Normalization of luciferase and statistical methods

The quantitative results are expressed as relative luminescence units (RLU) and are normalized by taking the ratios of Firefly/Renilla luciferase. To determine the relative luciferase activity (RLA), Firefly/Renilla ratios were first calculated for each individual sample from acquired luminometer data. Then a calculation using a formula from (Potter et al. 2010) was performed to determine RLA:

$$RLA_x = (F_x/R_x)/\ (\overline{F/R})_{Responder},$$

where

$$(\overline{F/R})_{Responder} = (\sum_{i=1}^{n} F^i_{Responder}\ /R^i_{Responder})/n,$$

Where n = number of Responder-only samples; F = Firefly luciferase RLU; R = Renilla luciferase RLU. The average and SEM were determined for each treatment and statistical

significance was determined using a Student's t test. Comparisons were considered statistically significant with $p<0.05$. The software used for these analyses was GraphPad Prism version 8.3.1 for macOS (GraphPad Software, San Diego).

5.3.8 Transactivator modelling

The tertiary structure of the binary proteins were modeled using LOMET online, a meta server based protein fold recognition (Zheng et al. 2019; Wu and Zhang 2007). With this online tool were generated 3D models by collecting high-scoring structural templates threading that were compared with the crystal structure of repressor proteins characterized in the literature.

5.4 Results

5.4.1 Design and development of additional binary systems in fruit flies

To characterize the utility of bacterially derived transactivation systems in D. melanogaster, including: the p-CymR operon from Pseudomonas putida (Mullick et al. 2006), the PipR operon from Streptomyces coelicolor (Fussenegger et al. 2000), the TtgR operon from Pseudomonas putida (Gitzinger et al. 2009), the VanR operon from Caulobacter crescentus (Gitzinger et al. 2012), we designed a dual luciferase reporter system utilizing the repressor and corresponding operator sequences from each bacterial operon. The widely used TetR system served as a positive control (Bello, Resendez-Perez, and Gehring 1998). Separate "driver" and "responder" transgenic lines were generated that could be genetically crossed to visualize and quantify transactivation responses (Figure 5.1A, Figure S4.1A). In each driver line, a flightin (Fln) promoter fragment (Ayer and Vigoreaux 2003) was used to drive expression of a chimeric transactivator in the indirect flight muscles consisting of the operon specific repressor protein (i.e.

CymR, PipR, TtgR, VanR, or TetR) fused to three tandem VP16 activation domains (Das, Tenenbaum, and Berkhout 2016; Wysocka and Herr 2003)(Figure 5.1B, Figure S5.1A). Each responder line was designed with 2-3 operator sequences, specific to each operon (i.e. CymO, PipO, TtgO, VanO, or TetO) and upstream of both a minimal Hsp70 (Amin et al. 1987) and a UASp (Rørth 1998) promoter driving expression of firefly luciferase reporter genes linked to a T2A-GFP marker to enable direct quantification and visualization of transactivation via luciferase and GFP, respectively. The construct was terminated by a baculovirus derived p10 3' UTR known to increase efficiency of both polyadenylation and expression (Pfeiffer, Truman, and Rubin 2012) (Figure S5.1A). Ubiquitously expressed renilla luciferase with a SV40 3'UTR was also added to the responder construct, oriented in the opposite direction, to enable the normalization of firefly luciferase expression from the same genomic context. Both the driver and responder constructs were marked with the mini-white transformation marker (Pirrotta 1988), and inserted using phiC31 site-specifically into the same 3rd chromosomal site as the test system to enable direct comparisons (Figure 5.2A). Transgenic flies were balanced and maintained as homozygous stocks.

5.4.2 Binary systems as transactivators for gene expression

To determine whether these bacterial systems were capable of transactivating reporter genes in flies, we first performed a genetic cross between the driver and responder lines to produce transheterozygotes (Figure 5.2A). For each transhetrozygous transactivator/responder combination, robust GFP fluorescence was visible in the adult thorax where the flightN promoter was expressed in the indirect flight muscles, indicating that each combination was robustly transactivating (Figure 5.2B). Importantly, no basal GFP expression was observed in the control flies, which only harbored either the driver or responder, but not both. Differences in GFP

fluorescence intensity across all systems, indicated system-specific differences in the levels of reporter gene expression, despite the fact that all the driver and responder transgenes were located on the same chromosomal site. Specifically, the CymR/CymO (cymTA) system had the highest visible GFP fluorescence, while the tetR/TetO (tTA) and the VanR/Van (vanTA) systems had the lowest (Figure 5.2B). Because GFP fluorescence only provides a visual qualitative confirmation of transactivation, we next measured luciferase expression for an accurate quantification. To do this, both firefly and renilla luciferase were measured in 3–day-old transheterozygous individual flies using a dual luciferase assay (Figure 5.2A). In all systems, transheterozygous flies had significant activation of firefly luciferase compared to control responder-only flies, suggesting robust transactivation for each system (Figure 5.3B) (all systems had at least a $p \leq 0.005$).

5.4.3 Suppression of reporter genes in binary systems

Similar to the Tet-OFF system repressor, which interacts with tetracycline and doxycycline, the repressors from the cymTA, pipTA, ttgTA, and vanTA systems interact with their own specific ligands, corresponding to cumate, virginiamycin M1, phloretin, and vanillic acid, respectively (Mullick et al. 2006; Fussenegger et al. 2000; Gitzinger et al. 2009, 2012)(Figure S5.1B). Leveraging this prior work, we hypothesized that when no ligand is present, the transactivator should bind to its operon, promoting spatial expression of the reporter genes in the flight muscles (Figure 5.2). However, when ligand is present and bound to the transactivator, this should result in a confirmational change and temporally prevent the transactivator from binding to its operator. Therefore, the absence of ligand, termed the OFF configuration, should enable the measurement of the maximum spatial gene expression levels of these systems, while the presence of ligand should temporally reduce expression (Figure 5.3B). To assess this potential, we measured

the ability of a small-molecule ligand to repress gene expression in each system. We used three initial ligand concentrations, low dose (0.5-10µg/mL), high dose (5-100 µg/mL), and very high dose (50-1,000µg/mL), though the very high dosage of ligand proved too toxic for fly survival and was excluded. Transheterozygous tTA flies reared on a low dose (1µg/mL) and high dose (10µg/mL) of doxycycline showed a concentration-dependent decrease in GFP fluorescence and firefly luciferase expression (Figure 5.2B and Figure 5.3B) ($p \leq 0.0005$ and $p \leq 0.0002$, respectively). However, we did not detect a concentration-dependent decrease in luciferase expression in cymTA, pipTA, ttgTA, and vanTA flies (Figure 5.3B). Confirming this lack of system repression in the presence of the ligand, our fluorescence images indicated that the GFP levels also remained constant for these systems (Figure 5.2B). This suggests that the highest testable concentrations for each compound (10x) were not sufficient to suppress the cymTA, pipTA, ttgTA, and vanTA systems and may reflect a pharmacokinetics issue of the ligands not reaching the indirect flight muscle and would be worth testing these systems in other tissues in vivo in the future.

5.5 Discussion

In this study, we evaluate four bacterially derived transactivation systems in vivo in Drosophila melanogaster. The use of transgenic binary systems to temporally and spatially control gene expression is one of the most powerful tools in synthetic molecular biology, and these systems assist researchers in modifying cellular functions, generating cellular responses to environmental stimuli, and influencing cellular development (Lewandoski 2001). While established spatial-temporal control systems like GAL4-UAS, Tet-OFF, and the Q-system exist, generating additional systems for the Drosophila tool box will be crucial for selectively choosing systems for desired

functions or applications. In our work, we demonstrate cymTA, pipTA, ttgTA, and vanTA each robustly and spatially transactivate gene expression in fruit flies, providing new binary transactivation systems that can be used for various research applications.

Using our quantitative luciferase assay, each system was able to demonstrate a higher level of transactivation when compared to the TtA system. This result is promising because higher induction expression systems can be used in several fruit fly applications. Robust expression is generally a desired feature in the development of binary systems. While the tested systems proved to be stronger than TET-OFF, there are still expression level differences between them. For example, vanTA demonstrated the highest average RLU (~360), with both cymTA and pipTa averaging around ~150 RLU, and ttgTA having the lowest average (~75). These differences in gene expression enable the user to choose their desired expression level output (low to high). Even without system repression using their corresponding ligands, these systems still function well for binary gene transactivation.

Since these systems are highly efficient in cell culture (Gitzinger et al. 2009, 2012; Mullick et al. 2006; Fussenegger et al. 2000), they should still be capable of small-molecule control in vivo, despite the lack of control we observed in the flight muscle. The Tet-OFF (tTA) positive control suggested our experimental design was sufficient for the activation and suppression of reporter genes. Therefore, it is possible that the amount of compound fed to the animal was not sufficient to either (1) reach the indirect flight muscle tissues or (2) fully suppress the system. The first hypothesis was proposed because in order for the compound to reach the flight muscles, it must pass the midgut and travel through the hemolymph and likely through other organs before reaching the target tissue. To test this hypothesis, the transgenes need to be re-designed to enable expression of reporter genes in the midgut or another easily accessible tissue, where the ligand could more

easily reach its target transactivator. Cell culture studies suggest these ligands are able to cross the cell membrane, which indicates these ligands should also be capable of entering animal cells in vivo. Whether the ligand is metabolized by the insect, however, is unknown, though could explain the lack of suppression. A comprehensive pharmacokinetics assay may resolve these unknowns.

Our second hypothesis for the lack of ligand repression is that we were unable to reach a concentration that would fully repress the system. In tTA, doxycycline at the 10x concentration was able to fully suppress the system in flies, which may be due to the comparatively lower gene activation level of this system. The concentration of doxycycline tested was sufficient to prevent the transactivator from binding to the operator sequence. Due to the higher levels of transactivation observed in the cymTA, pipTA, vanTA, and to some extent ttgTA systems, it is probable that higher ligand concentrations will be needed for suppression. Because a higher concentration via oral feeding was highly toxic to flies, it would be difficult to conduct such an experiment in a live animal model. Higher concentrations may be achieved via thoracic injection, however, this may be impractical for experiments where tissues are difficult to reach or may otherwise kill the animal.

Taken together, we conclusively demonstrated that these bacterially-derived systems can robustly function as genetic binary transactivational systems in vivo and these tools expand the molecular genetic *Drosophila* toolbox. Our work provides the first step in the characterization of new transactivation systems in fruit flies and may contribute to the generation of novel synthetic tools that can be used in other animal systems, perhaps even mosquitoes (Zhao, Tian, and McBride 2020) to elucidate molecular genetic questions and to design advanced biological circuits.

5.6 Acknowledgments

This chapter, in full, is a copy of the material that is currently being prepared for publication. Stephanie Gamez, Luis C. Vesga, Stelia C. Mendez-Sanchez, Omar S. Akbari. "Development of alternative transactivational binary expression systems in *Drosophila melanogaster*." The dissertation/thesis author was first author and primary investigator of this paper

5.7 Figures

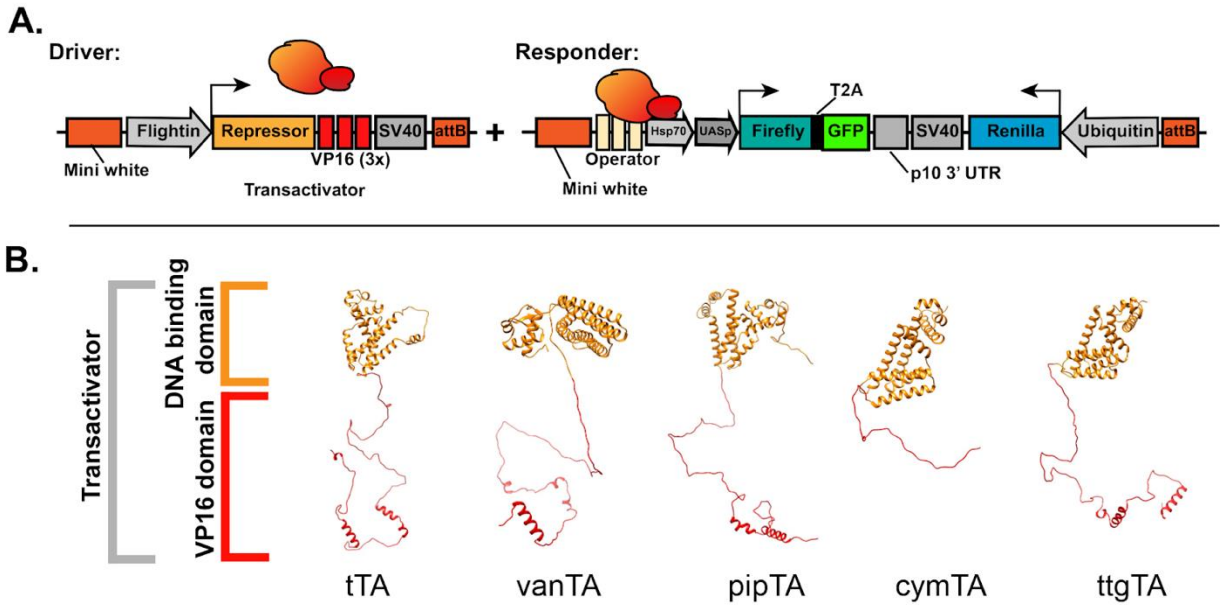


Figure 5.1: Characterization of transactivation systems. (A) Schematic of the general components in "driver" and "responder" transgenes. Driver transgenes are composed of a regulatory element driving the expression of a repressor fused to three tandem VP16 activation domains (transactivator). The responder transgene contains an operator sequence (2-3 copies), minimal promoters (HSP70 and UASp), firefly and renilla luciferase, and GFP to visualize and quantify transactivation. The transactivator (depicted in orange-red) binds to the operator sequence and induces the expression of reporter genes. A ubiquitous renilla luciferase in the responder transgene enables normalization of firefly luciferase. (B) 3D structural protein models (homology models) of system transactivators used in this study. Transactivators are made up of the DNA binding domain (shown in orange) and three tandem VP16 activation domains (in red). For each system, protein modeling was used to depict overall folding of fused transactivational components.

Figure 5.2: Characterization of alternative binary systems in Drosophila melanogaster. (A) Driver and responder transgenes were inserted site-specifically using a phiC31/attP docking site (ZH-attP-86Fa). A genetic cross between the homozygous driver and responder strains produced transheterozygous flies all displaying robust GFP fluorescence in the adult indirect flight muscles. Male flies were collected for imaging and quantification of dual luciferase reporters. (B) Transactivation of the GFP reporter was observed in all transheterozygotes in both the absence or presence of ligand. Robust GFP expression was observed in all transheterozygous (driver + responder) flies, while no GFP expression was observed in control files including wildtype, or driver-only, or responder-only files. White arrows point to the fly thorax/flight muscles where Fln is expressed. The ligand used is listed vertically on the right of each image. The ligand concentration fed to flies is indicated in the top right-hand corner of each image. Genotypes are shown in the bottom left corner. Only 1-2 day old males were imaged.

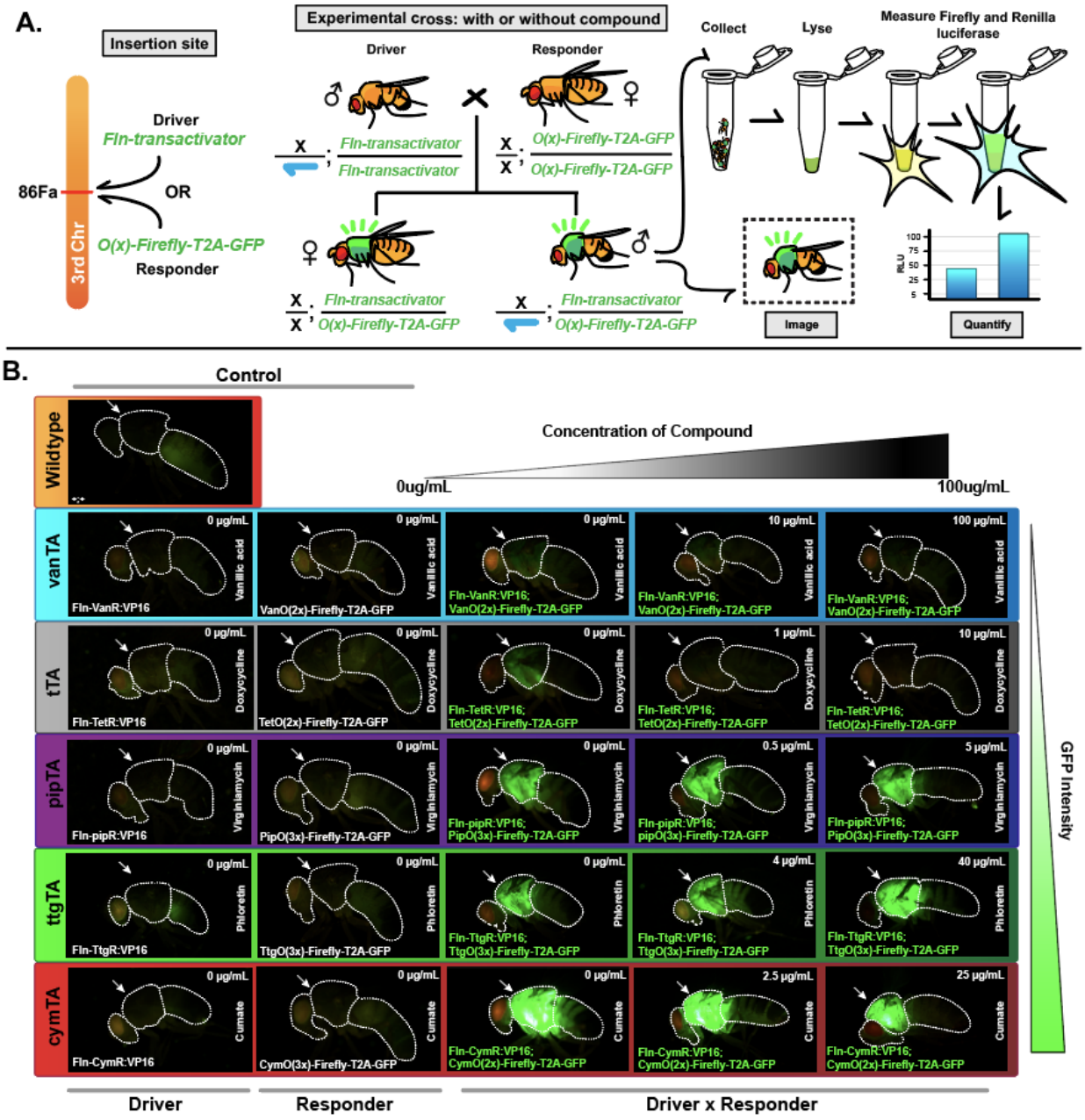
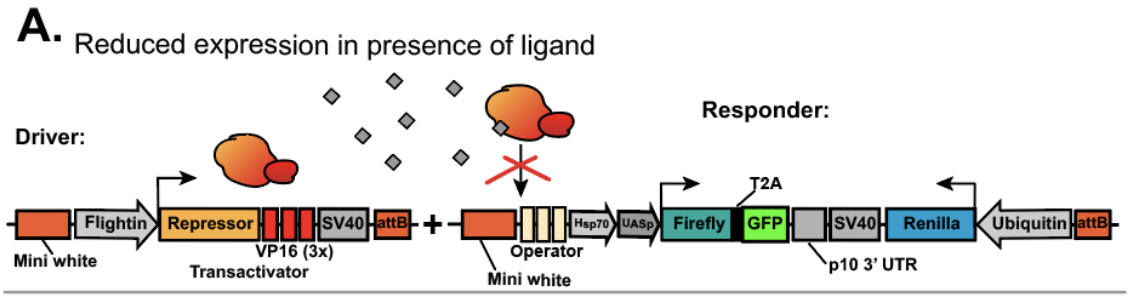
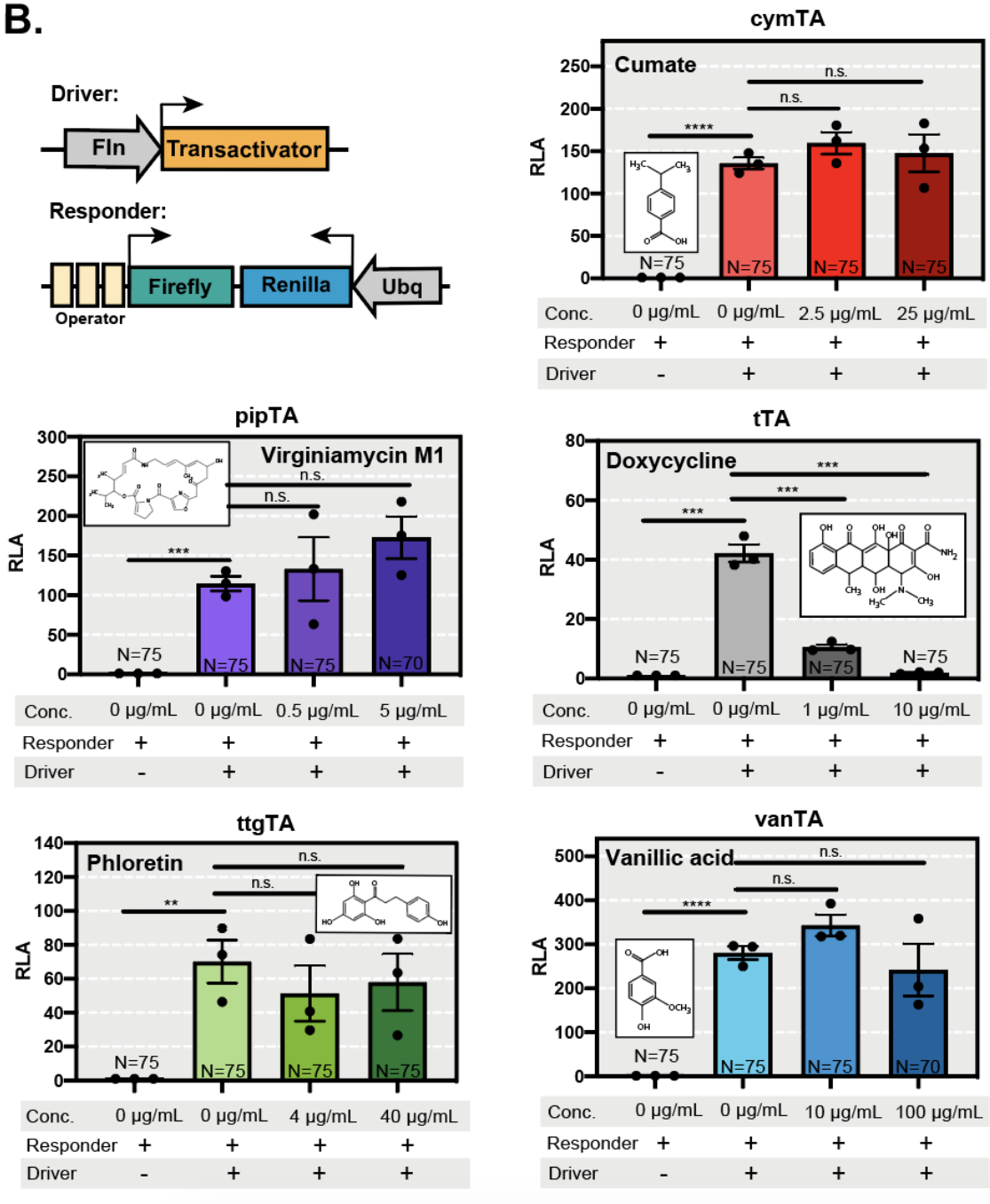

A.
Insertion site
86Fa
3rd Chr
Driver
FlnA-transactivator
OR
O(x)-Firefly-T2A-GFP
Responder
Experimental cross: with or without compound
Driver
Responder
Collect
Lyse
Measure Firefly and Renilla luciferase
Image
Quantify
B.
Control
Wildtype
Concentration of Compound
0ug/mL
100ug/mL
vanTA
tTA
pipTA
ttgTA
cymTA
GFP Intensity
Driver
Responder
Driver x Responder

Figure 5.3: Transcriptional activity of transgenic flies with or without ligands. (A) We tested whether expression of reporter genes in the responder transgene depended on the presence or absence of ligand. In the absence of a ligand, the transactivator (depicted in orange-red) binds to the operator sequence and induces the expression of reporter genes. In the presence of a ligand (depicted as a gray diamond), we expected the ligand binding to the transactivator to prevent the transactivator from binding to the operator sequence, preventing reporter gene expression. (B) Relative luciferase activity (RLA) for responder-only flies (control) and transheterozygous flies on increasing concentrations of ligand. All systems show significant transactivation of firefly luciferase expression when both driver and responder transgenes are present in the same genomic context. Each system displays a unique expression level, depicting system-specific differences. When cymTA, pipTA, ttgTA, and vanTA transheterozygous flies were reared on food containing a low dose (0.5-10 µg/mL) or a high dose (5-100 µg/mL) of ligand (indicated on the top left-hand side of the plot), no significant reduction of luciferase activity was measured. Only tTA, the positive control, showed a concentration-dependent reduction of luciferase activity. Each dot represents one biological replicate composed of the average of 5 sub-replicates. Exceptions include vanTA and pipTA, where one sub replicate (out of five sub replicates) in one of the high dose treatments (out of 3 replicates) could not be collected due to the difficulty of obtaining sufficient number of transheterozygous flies on their treatments. N represents the total number of flies tested. Bars represent the standard error of the mean (SEM). Significance was determined using a student's t test. ****$p < 0.0001$; ***$p < 0.0002$; **$p < 0.0005$; n.s. not significant.


A.
Reduced expression in presence of ligand
Driver:
Responder:
Flightin
Repressor
SV40
attB
Mini white
VP16 (3x)
Transactivator
Operator
Mini white
Hsp70
UASp
Firefly
T2A
GFP
SV40
Renilla
Ubiquitin
attB
p10 3' UTR


B.
Driver:
FlnTransactivator
Responder:
Firefly
Renilla
Ubq
Operator
cymTA
Cumate
RLA
n.s.
n.s.
****
N=75
N=75
N=75
N=75
Conc. 0 µg/mL 0 µg/mL 2.5 µg/mL 25 µg/mL
Responder + + + +
Driver - + + +
pipTA
Virginiamycin M1
RLA
n.s.
n.s.
***
N=75
N=75
N=75
N=70
Conc. 0 µg/mL 0 µg/mL 0.5 µg/mL 5 µg/mL
Responder + + + +
Driver - + + +
tTA
Doxycycline
RLA
***
***
***
N=75
N=75
N=75
N=75
Conc. 0 µg/mL 0 µg/mL 1 µg/mL 10 µg/mL
Responder + + + +
Driver - + + +
ttgTA
Phloretin
RLA
n.s.
n.s.
**
N=75
N=75
N=75
N=75
Conc. 0 µg/mL 0 µg/mL 4 µg/mL 40 µg/mL
Responder + + + +
Driver - + + +
vanTA
Vanillic acid
RLA
n.s.
n.s.
****
N=75
N=75
N=75
N=70
Conc. 0 µg/mL 0 µg/mL 10 µg/mL 100 µg/mL
Responder + + + +
Driver - + + +

5.8 Supplemental Figures

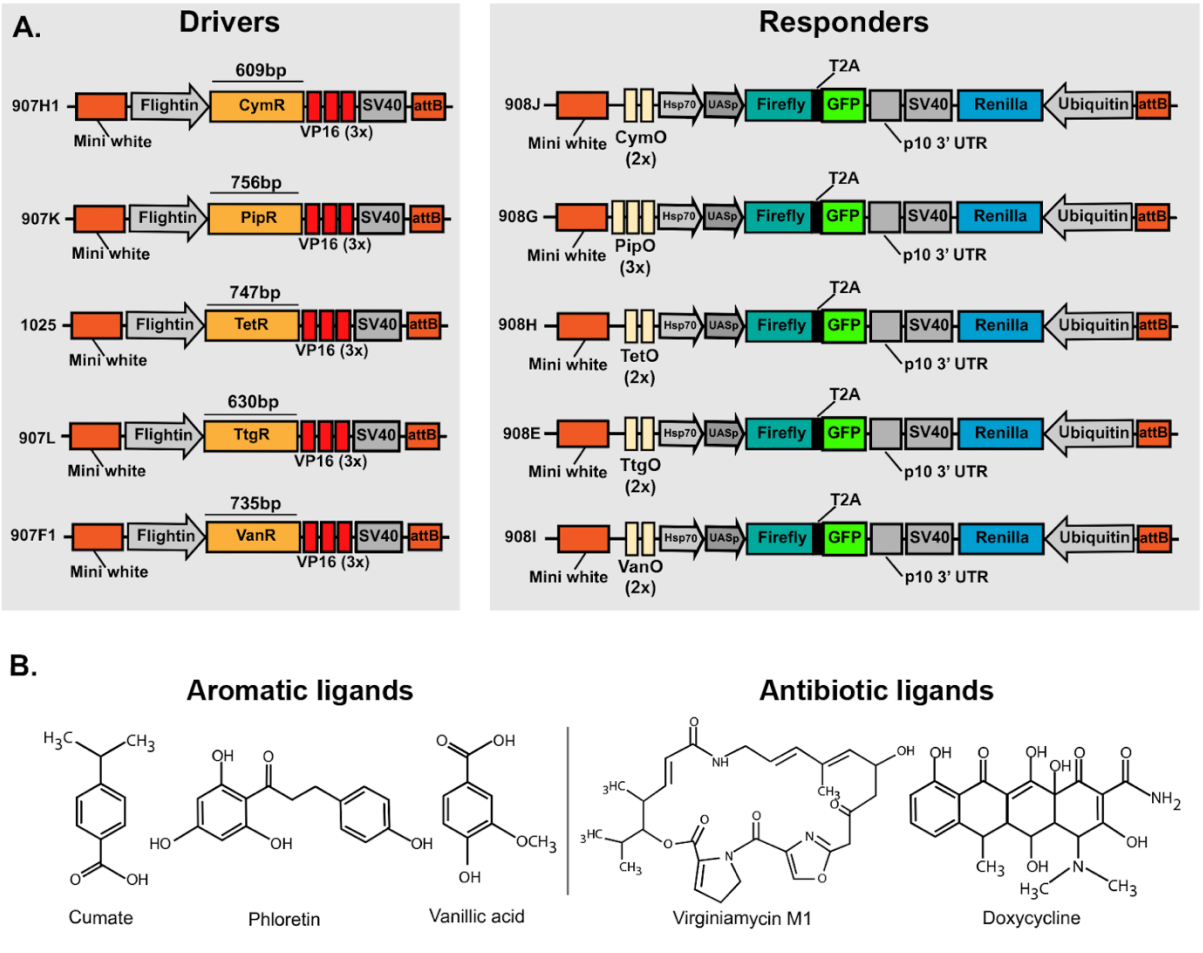


Figure S5.1: Schematic representation of driver and responder transgenes tested for each system. (A) Each regulation system described in this study is distinguished by its repressor sequence, operon sequence, and ligand used to suppress the system. To compare all these systems in Drosophila melanogaster, the rest of the components were kept consistent between transgenes. (B) Chemical structures of ligands tested in this study. These ligands can be characterized as aromatic or antibiotic.

5.9 Supplemental Tables

Table S5.3: Final vector constructs and their descriptions. List of vectors and transgenic fly lines generated in this study.

Vector #	Addgene ID	BSC ID	FlyBase symbols	Description
907F1	160233	91379	w[*];M{RFP[3xP3.PB] w[+mC]=fln-vanTA.G}ZH-86Fa	mini-w+-FlightN-vanTA-SV40-attB
907H1	160234	TBD	M{fln-cymTA.G}ZH-86Fa	mini-w+-FlightN-cymTA-SV40-attB
1025	160235	91381	w[*];M{RFP[3xP3.PB] w[+mC]=fln-tTA.G}ZH-86Fa	mini-w+FlightN-tTA-SV40-attB
907K	160236	91380	w[*];M{RFP[3xP3.PB] w[+mC]=fln-pipTA.G}ZH-86Fa	mini-w+-FlightN-pipTA-SV40-attB
907L	160237	TBD	M{fln-ttgTA.G}ZH-86Fa	mini-w+-FlightN-ttgTA-SV40-attB
908E	160238	91382	w[*];M{RFP[3xP3.PB] w[+mC]=ttgO-UASp-pLUC.T2A.EGFP,Ubi-rLUC}ZH-86Fa	mini-w+-ttgO(2x)-Hsp70min-UASpmin-Firefly-T2A-eGFP-p10-3'UTR-SV40-Ubiquitin-Renilla-attB
908G	160239	91383	w[*];M{RFP[3xP3.PB] w[+mC]=pipO-UASp-pLUC.T2A.EGFP,Ubi-rLUC}ZH-86Fa	mini-w+-pipO(3x)-Hsp70min-UASpmin-Firefly-T2A-eGFP-p10-3'UTR-SV40-Ubiquitin-Renilla-attB
908H	160240	91384	w[*];M{RFP[3xP3.PB] w[+mC]=tetO-UASp-pLUC.T2A.EGFP,Ubi-rLUC}ZH-86Fa	mini-w+-tetO(2x)-Hsp70min-UASpmin-Firefly-T2A-eGFP-p10-3'UTR-Ubiquitin-Renilla-attB
908I	160241	91385	w[*];M{RFP[3xP3.PB] w[+mC]=vanO-UASp-pLUC.T2A.EGFP,Ubi-rLUC}ZH-86Fa	mini-w+-vanO(2x)-Hsp70min-UASpmin-Firefly-T2A-eGFP-p10-3'UTR-SV40-Ubiquitin-Renilla-attB
908J	160242	TBD	M{cymO-UASp-pLUC.T2A.EGFP,Ubi-rLUC}ZH-86Fa	mini-w+-cymO(2x)-Hsp70min-UASpmin-Firefly-T2A-eGFP-p10-3'UTR-SV40-Ubiquitin-Renilla-attB

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