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UNIVERSITY OF CALIFORNIA
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The Role of MicroRNAs During the Gonadotrophic Cycles of the
Female Mosquito, *Aedes aegypti*

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

Doctor of Philosophy

in

Genetics, Genomics and Bioinformatics

by

Keira Justine Lucas

June 2015

Dissertation Committee:

Dr. Alexander S. Raikhel, Chairperson

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The Dissertation of Keira Justine Lucas is approved:

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University of California, Riverside

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ABSTRACT OF THE DISSERTATION

The Role of MicroRNAs During the Gonadotrophic Cycles of the
Female Mosquito, *Aedes aegypti*

by

Keira Justine Lucas

Doctor of Philosophy, Graduate Program in Genetics, Genome and Bioinformatics
University of California, Riverside, June 2015
Dr. Alexander S. Raikhel, Chairperson

Female mosquitoes require a blood meal for reproduction, providing the underlying mechanism for the spread of many devastating parasitic and viral vector-borne diseases in humans. A deeper understanding of the molecular mechanisms linked to mosquito blood meal processes, reproductive events and anti-pathogen immunity is of particular importance for devising innovative vector control strategies. Acquisition of blood initiates a cascade of events – including dynamic changes in microRNA (miRNAs) expression – in various tissues in the female mosquito. miRNAs are a class of endogenous regulatory RNA molecules 21-24 nucleotides in length that modulate gene expression at the post-transcriptional level via base pairing to target sites within messenger RNAs (mRNA). Reports have indicated that miRNAs are differentially expressed in various tissues of the female mosquito upon the uptake of a blood meal. miRNAs have been shown to play important roles in regulation gene expression throughout the adult mosquitoes life cycle – including the regulation of adult development, blood digestion and mosquito-pathogen interaction. Furthermore, several

lineage-specific miRNAs have been identified in mosquitoes and may underlie mosquito-specific events, including blood meal-initiated events. This thesis describes the fat body specific function of the conserved miRNA, miR-8, in the female mosquito post-blood meal and its involvement in regulating essential reproductive events through targeting the Wingleless signaling pathway. Additionally, this thesis details the function of two lineage-specific miRNAs, miR-1174 and miR-1890, and their involvement in regulating digestive processes in the female mosquito midgut. Together, these studies have established a fundamental role for both conserved and lineage-specific miRNAs in the adult female mosquito.

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CHAPTER 1

MicroRNAs: A New Frontier in Mosquito Biology

1.1 Introduction to mosquito biology

Females of the anautogenous mosquito species serve as major vectors of many medically important arboviral and parasitic diseases due to their need for vertebrate blood for egg production and their cyclic blood feeding habits. These vector-borne diseases result in severe global health and economic burdens worldwide. Since the 1970's epidemics of malaria, dengue, and other formerly contained vector-borne diseases have been on the rise in the developing world. *Aedes aegypti* is the major vector for many debilitating arboviruses, including yellow fever, dengue (DENV) and chikungunya (CHIKV) virus. The global incidence of DENV has increased dramatically in recent years, with over one-third of the world's population at risk of DENV infection and an estimated 390 million infections annually (1). CHIKV has spread to the Americas at an explosive rate, with over 1 million reported cases since its emergence in 2013 (2). *Anopheles gambiae* is the vector of the most deadly form of malaria, *Plasmodium falciparum*, and caused an estimated 584,000 deaths in 2013, mostly in children under five years of age (3). Despite significant efforts, controlling these diseases and their vectors remains challenging. Transmission of disease pathogens by female mosquitoes is tightly linked to blood feeding which, in turn, is required for reproduction. Hence, deciphering the mechanisms that govern the major functions linked to the female mosquito's ability to utilize blood and develop eggs is of paramount importance.

The mosquito fat body, an adipose tissue analogous to the mammalian liver, is an important center for energy metabolism, immunity and reproduction. Central to mosquito reproduction is the highly regulated process of vitellogenesis, in which a protein rich blood meal initiates the synthesis and secretion of yolk protein precursors (YPPs) by the fat body (4). Activation of vitellogenesis requires many intricate hormonal and nutritional cues, including insulin, ecdysone and the Target of Rapamycin (TOR) nutrient sensor (4, 5). In response to a blood meal, two types of steroidogenic hormones, ovary ecdysteroidogenic hormone I (OEH I) and insulin-like peptides (ILPs), are secreted from the brain, stimulating the ovaries to secrete ecdysone, which is then converted to 20E in the fat body (5-7). Crosstalk between the TOR and insulin signaling pathways, along with production of 20E in the fat body, work to regulate YPP gene expression. Amino acid (AA) signaling through the TOR pathway and 20E stimulation enhances YPP gene expression. YPPs are then synthesized in the fat body, secreted into the hemolymph and taken up by the developing oocytes through receptor-mediated endocytosis, where they are converted to mature yolk proteins and deposited in yolk granules (8). These yolk proteins provide essential nutrients vital for embryonic development. Due to the fat body's essential role in producing and secreting the YPPs necessary for egg development, it has received substantial attention in developing a better understanding of mosquito reproductive process. In addition, the female mosquito gut serves as the central organ for the digestion of blood and liberation of AA used by the fat body for activation of vitellogenesis. Studying the molecular events within these vital tissues is essential to identify important regulators of mosquito blood digestion and reproductive processes.

To date, major research efforts have been focused on *Ae. aegypti* and *An. gambiae*; mainly due to their medical importance as disease vectors, availability of well-annotated genome sequences, enhanced bioinformatic resources and advances in genetic tools for molecular studies (9-15). Until recently, systemic approaches to knockdown gene products through microinjection were driving studies of individual gene function. However, the establishment and continuous efforts to advance the *Ae. aegypti* and *An. gambiae* Gal4-UAS systems now provides a tool to assess gene function with spatiotemporal resolution (9-11). Recent investigations of the CRISPR-Cas9 system in *Ae. aegypti* offers promising results that will allow genome engineering previously out of reach for non-model organisms (12-14). Furthermore, genome-wide transcriptomic studies of *Ae. aegypti* and *An. gambiae* have revealed repertoires of genes involved in physiological events linked to blood feeding and female reproduction (16-19).

1.2 Introduction to RNA silencing and microRNAs

The discovery of RNA interference (RNAi) and small non-coding RNAs (sRNA) represents one of the most significant breakthroughs in molecular biology. RNAi is an evolutionarily conserved mechanism of sequence specific gene silencing that is triggered by endogenous or invading double stranded RNAs (dsRNA) arising from hairpin structures, transposable elements, or viral infections. The finding that small dsRNA molecules control gene expression has significantly altered our understanding of gene regulatory networks. Further, the development of RNAi technology in insect species provides a key resource for investigating gene functions in non-drosophilid insects where

genetic mutants are unavailable. Much of what is known about sRNA function in insects is based on studies in the fruit fly, *Drosophila melanogaster*. However, as genetic tools required for the study of sRNAs become established in non-drosophilid insects, more information regarding the function of sRNAs is becoming available in these organisms.

The RNAi pathway in animals includes several branches that function in silencing endogenous genes, RNA viruses, and mobile genetic elements. The three classes of regulatory sRNAs in animals include: microRNAs (miRNA), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). These regulatory sRNAs are categorized based on their size and interaction with particular types of Argonaute (Ago) proteins. Typically in insects, 22 nucleotide (nt) miRNAs are loaded into Ago-1, 21 nt siRNAs interact with Ago-2 containing effector complexes, and 24-31 nt piRNAs associate with the Piwi-subfamily of Ago proteins.

The first implication of RNA silencing was discovered in an attempt to overexpress a pigment synthesis enzyme, *chalcone synthase*, in order to produce a deep purple petunia; instead, the large majority of the plants harboring the *chalcone synthase* transgene produced completely white flowers (20, 21). This phenomenon, coined “co-suppression”, pioneered the way to understanding RNA silencing. Studies in *Caenorhabditis elegans* indicated that the expression of both sense and anti-sense RNA strands could lead to effective inhibition of target genes (22, 23). However, the key finding that defined the RNAi pathway was the discovery that dsRNA created to target exonic regions of the genome produced efficient depletion of the gene product (24). Soon after, it was identified that 20-25 nt siRNAs derived from either naturally occurring or

foreign dsRNAs served as the basis of post-transcriptional gene silencing (25). RNAi was subsequently shown to work successfully in *Drosophila*, where it was used to investigate the functions of *frizzled* and *frizzled-2* and determine their action in the Wingless (Wg) signaling pathway (26).

The first miRNA was discovered in *C. elegans* by the identification of two transcripts arising from the *lin-4* locus: the 22 nt *lin-4s* and the 61 nt *lin-4l* (27, 28). It was shown that translation of *lin-14* is regulated by *lin-4* by its 3' untranslated region (UTR) through an anti-sense mechanism. It was not until 7 years later that the next miRNA was discovered, in which the 21 nt *let-7* in *C. elegans* was shown to temporally regulate *lin-4l* by binding target sites within its 3' UTR (29). Since the discovery of *lin-4* and *let-7* thousands of putative miRNAs have been computationally and experimentally identified in various organisms.

1.3 MicroRNA biogenesis in insects

RNA polymerase III (pol III) was initially thought to mediate the transcription of most miRNA loci because of its role in the transcription of short non-coding RNAs such as transfer RNAs and U6 small nuclear RNAs. However, miRNA gene structure and direct experimental evidence suggests that RNA polymerase II (pol II) is the main RNA polymerase mediating the transcription of primary miRNAs (pri-miRNA) in animals. Although most miRNAs are derived from intergenic regions and are characterized as independent transcription units, some miRNA genes are located in intronic regions and are transcribed in parallel with their host transcript by pol II (30). In addition, there are

several miRNA genes that exist in clusters and are transcribed as one bicistronic or polycistronic pri-miRNA transcript (31). The majority of pri-miRNA transcripts also contain traditional 5' 7-methyl guanosine caps and 3' polyadenylation, indicative of pol II transcription (32).

Pri-miRNA transcripts typically fold into a ~30 bp stem structure with a terminal loop and flanking segments. These transcripts are processed in the nucleus into a 70 nt precursor miRNA (pre-miRNA) by the microprocessor complex, which recognizes the pri-miRNA and removes the single stranded flanking segments. The ~500 kDa microprocessor complex contains an RNase III enzyme, Drosha, and its dsRNA binding partner Pasha (DGCR8 in mammals and *C.elegans*) (33-36). Pasha recognizes the substrate pri-miRNA, anchors to the flanking single-stranded RNA (ssRNA) and dsRNA stem junction, and locates the position 11 bp into the stem where the processing center of Drosha is placed to cleave the pri-miRNA (37).

After processing of the pri-miRNA into a 70 nt pre-miRNA, the pre-miRNA is exported into the cytoplasm for further modifications. A Ran guanosine triphosphate (RanGTP)-dependent dsRNA-binding receptor, Exportin-5 (Exp-5), mediates the nuclear export of pre-miRNAs by recognizing the 2 nt 3' overhang of the pre-miRNA produced by the microprocessor complex (38-40). Exp-5 not only serves as a nuclear export factor, but it also protects pre-miRNAs from digestion by harmful nucleases (39, 40). Together with Exp-5, the pre-miRNA migrates through nuclear pore complexes into the cytoplasm, where the release of the pre-miRNA occurs in response to the hydrolysis of RanGTP to RanGDP.

In the cytoplasm, the terminal loop structure of the pre-miRNA is cleaved by another RNase III enzyme, Dicer-1 (Dcr-1), relieving a ~22 nt miRNA duplex (41-43). Like Drosha, Dcr-1 also requires a dsRNA binding partner, known as Loquacious (Loqs), in order to exert its action on pre-miRNAs (44, 45). Dcr-1 determines the authenticity of a pre-miRNA by recognizing the pre-miRNA loop structure through its N-terminal helicase domain and proceeds to measure the loop size. In addition, the Dcr-1 PAZ domain recognizes the 2 nt 3' overhang produced by the microprocessor complex and measures the distance from the overhang to the single stranded terminal loop (46).

Upon Dcr-1 cleavage of the pre-miRNA to form the mature miRNA-miRNA* duplex, the duplex strands are sorted and loaded into the RNA induced silencing complex (RISC). The core component of RISC is the Ago family of sRNA guided RNA-binding proteins. The *Drosophila* genome encodes five distinct Ago proteins, which are categorized into two sub-clades: Ago and Piwi. The Ago sub-clade comprises Ago-1 and Ago-2, which were originally believed to bind miRNAs and siRNAs, respectively (47, 48). It was thought that the mature miRNA guide strand was loaded into Ago-1 while the miRNA* passenger strand was degraded; however, studies suggest that certain characteristics of the miRNA-miRNA* duplex influence strand selection and partitioning between Ago-1 and Ago-2 (49-51). Sorting of miRNAs into Ago1-RISC is influenced by a 5' uracil and thermodynamic instability caused by central bulges at nucleotide 7-11, while sorting into Ago2-RISC is sensitive to thermodynamic stability, including base pairing at nucleotides 9-10. Typically, miRNA-Ago complexes silence gene expression post-transcriptionally through translational inhibition and subsequent mRNA degradation

(52-54). This occurs through imperfect base-pairing to target “seed match” sites within the 3' UTR of mRNAs (27, 28). Functional binding sites of miRNAs have also been identified in the 5' UTR (55) and open reading frame (56-58) of mRNAs as well.

1.4 Tools for studying microRNA functions in non-model organisms

In *Drosophila*, classic genetic knockouts have been influential for the study of miRNA function. The first *Drosophila* miRNA mutant reported was *mir-14* (59), and since that time many miRNA loss-of-function or gain-of-function mutants have been used to investigate the roles of miRNAs in flies. However, in the majority of insect species, technology for the efficient production of mutant libraries is not widely available.

For loss-of-function studies, systemic knockdown effects can be achieved using sequence-specific miRNA inhibitors. A powerful miRNA inhibition method is the use a cholesterol conjugated antisense RNA oligonucleotide, known as an antagomir (60). These antisense oligonucleotides work as competitive inhibitors of miRNAs by annealing to the mature miRNA after incorporation into RISC. Potent antagomirs are designed to harbor various modifications for stability (2' O-methylation and phosphorothioate backbone) and delivery enhancement (cholesterol group at the 3' end). Microinjection of antagomirs into female mosquitoes has proved efficient for miRNA functional characterization (61). While antagomirs are simple tools to silence specific miRNAs, they provide narrow applicability in assessing miRNA function due to the lack of spatial and temporal specificity. In addition, a major challenge in their use is their stability and delivery both *in vitro* and *in vivo* (60).

To achieve a spatiotemporal inhibition of a miRNA, a miRNA sponge (miR-SP) can be employed. The miR-SP contains tandemly arrayed sequences complementary to a miRNA with a mismatched bulge at positions 9-12 (62, 63). A miR-SP designed to interact with a particular miRNA competes with the miRNAs target to “soak up” the miRNA. The first *Drosophila* miR-SP was produced by developing a cassette containing 10 bulged miR-8 binding sites expressed through an enhancer trap combined with the Gal4-UAS system (62). Another type of transgenic miRNA inhibitor, shown to be effective in mammalian cells, is the Tough Decoy (TuD) RNA (64). TuD RNAs contain miRNA binding sites in single-stranded regions of short stem loops, presenting the sites for binding of miRNA associated RISC. The miR-SP and TuD RNA transgenic methods provide powerful tools to assess the temporal and spatial functions of miRNAs *in vivo*; however, development and testing of these transgenic lines can be time consuming (60, 63). The establishment of the *Ae. aegypti* and *An. gambiae* Gal4-UAS systems now provide a valuable tool to assess miRNA function with spatiotemporal resolution (9-11). Significant progress has been made in developing strong driver lines to investigate the tissue-specific action of a gene of interest in the midgut of *An. gambiae* (11), and midgut (10) and fat body (9) of *Ae. aegypti* female mosquitoes post blood meal (PBM).

It appears that in many cases the consequence of loss of individual miRNAs is likely to have subtle or no phenotypic effects. In these cases, gain-of-function experiments can be utilized in order to unravel the roles of miRNAs. In insects, a miRNA gain-of-function effect can be achieved using treatments with miRNA mimic RNAs (65), expression vectors or a Gal4-UAS system for the overexpression of pri-miRNA

transcripts (66, 67). MicroRNA expression can be mimicked by injecting or feeding insects mimic RNAs that are identical in sequence to miRNAs. These so called “miRNA mimics” are chemically synthesized dsRNAs that mimic mature endogenous miRNAs. Overexpression or a gain-of-function effect of a particular miRNA can be assessed in a spatiotemporal specific manner by expressing pri-miRNA transcripts using the Gal4-UAS system.

Various computational target prediction tools are available for the identification of miRNA targets (68-71). Reliable identification of miRNA targets is different from standard sequence similarity analysis and requires additional criteria to assess functional miRNA binding sites. Many miRNA target prediction programs assess miRNA-target interaction through a variety of criteria, including sequence complementarity of the miRNA seed region, complementary miRNA binding sites within the 5' end of the miRNAs, and thermodynamic stability of the interaction. Conservation of predicted miRNA-target interactions in closely-related species (such as *Ae. aegypti* and *An. gambiae*) is an important additional criterion, given the high level of sequence conservation of miRNAs. The majority of readily available computational miRNA target prediction tools are not adapted to non-drosophilid insect genomes and “in-house” target prediction programs may be a beneficial complement for identifying miRNA targets in these organisms. In addition, a BLAST search for transcripts containing miRNA “seed” match sites combined with free energy of folding algorithms have also proven useful to overcome this obstacle (72).

In addition to miRNA target prediction *in silico*, experimental miRNA target identification tools may serve as a complement to computational prediction programs. CLIP-seq technology is a prevalent method to define Ago interactions and experimentally identify Ago bound miRNAs and targeted mRNA sites. In Ago HITS-CLIP (High Throughput Sequencing of RNAs isolated by Crosslinking Immunoprecipitation), RNA binding proteins (RBP) are UV cross-linked to bound RNA, and Ago bound mRNA and miRNAs are co-precipitated with an Ago antibody followed by RNA-seq (73). Ago HITS-CLIP experiments can be performed *in vivo* or *in vitro*, allowing target identification of a particular temporal or spatial specific miRNA. Ago PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) is another *in vitro* CLIP-seq method used to identify Ago interaction sites (74). Photoactivatable nucleoside analogs are incorporated into cellular RNA of cultured cells, enhancing cross-linking of RBPs to bound RNA under exposure to UV light of 365nm, and like HITS-CLIP, cross-linked Ago-RNA complexes are immunoprecipitated and bound RNA is subjected to RNA-seq.

Transcript analysis on samples overexpressing or knocking down a miRNA can be used to identify miRNA targets; however, these methods do not account for the detection of miRNAs that cause translational inhibition. Proteomic analysis can also be used to find miRNA targets. Stable isotope labeling with amino acids in cell culture (SILAC), is a high-throughput method for quantitative proteomics, and can be used to assess the effect of a miRNA on the proteome by overexpressing or knocking down a

miRNA of interest (75-77). However, direct or indirect effects on a transcript or protein cannot be distinguished in these experiments.

1.5 Conserved and lineage-specific microRNAs in mosquitoes

While the majority of our understanding of miRNA expression and function come from studies performed in *Drosophila*, on-going efforts have identified miRNAs in several mosquito species. The presence of miRNAs in mosquitoes was first experimentally verified by a small scale cloning method that found a small number of miRNAs in *An. gambiae* (78) and *An. stephensi* (79). Since these studies, several large scale expression profiling efforts have been made to identify both conserved and mosquito-specific miRNAs in *Ae. aegypti* (80-82), *Ae. albopictus* (83, 84), *Culex quinquefasciatus* (84), *An. anthropophagus* (85) *An. stephensi* (82, 86) and *An. gambiae* (87). Analysis of genomic organization of miRNAs in *An. gambiae* revealed that around 68% of miRNAs are intergenic miRNA, with the remaining 32% showing an overlap with predicted transcripts often mapping to the coding (sense) strand (87). Among those, 25% of miRNAs were located in introns, whereas approximately 7% were mapped to exons. Furthermore, a recent annotation of miRNA loci in *Ae. aegypti* and *An. stephensi* has revealed that nearly 30% of miRNAs are clustered and 20% of miRNAs are in the intronic region of the genome (82).

The majority of miRNAs identified in mosquitoes are conserved miRNAs, characterized by high sequence conservation across highly divergent species. While conserved miRNAs may have conserved targets across highly divergent species, they

may also participate in non-conserved miRNA targeting that is specific to certain lineages (88). Furthermore, conserved miRNAs may display significant variability in spatial and temporal expression between species (88). The conserved let-7 miRNA family represents an example of non-conserved miRNA targeting, in which mammalian let-7 targets the oncogene HMGA2, while HMGA2 orthologs are not targeted by let-7 in worms and flies (88).

In addition to conserved miRNAs, lineage-specific miRNAs have been identified in mosquitoes. These miRNAs have the ability to influence phenotypic divergence among animal species with important roles in a variety of biological processes, while complementing the function of conserved miRNAs (88). Lineage-specific miRNAs in mosquitoes may underlie mosquito-specific events, including blood feeding and reproductive processes. The miR-1174/miR-1175 cluster is an example of a lineage-specific miRNA conserved in blood feeding mosquitoes. While miR-1175 is found in both blood feeding and non-blood-feeding mosquitoes, miR-1174 is conserved in the blood feeding mosquito species *An. gambiae*, *An. stephensi*, and *Ae. aegypti* but not found in the non-blood-feeding mosquito, *T. amboinensis* (80). Species-specific miRNAs have also been identified in mosquitoes, with a recent miRNA discovery and annotation study revealing a total of four *Culicinae*-specific and two *Anopheles*-specific miRNAs (82). Lineage- and species-specific miRNAs may prove useful in the development of novel mosquito control strategies that allow targeting in species-specific manner.

1.6 MicroRNA regulation of mosquito blood digestion and egg development

Several miRNA library and functional analyses have provided clues to the regulation and function of miRNAs during mosquito blood digestion and ovarian development (61, 72, 78-80, 84, 87). Differential expression of miRNAs PBM indicates functions related to mosquito reproduction and blood-meal processes. The effect of blood-feeding on miRNA expression in the *An. gambiae* midgut resulted in an induction of miR-34, miR-317, and the mosquito-specific miR-1174/miR-1175 miRNA cluster (78). An expression analysis of miRNAs in the *Ae. aegypti* blood-fed midgut has also indicated that the majority of miRNAs are induced after blood feeding by 24 hours PBM (80). More recently, in *An. gambiae* whole body samples miR-7 was shown to be significantly induced, while miR-317 and a novel miRNA, miR-N3, were decreased 3 hours PBM (87). Furthermore, 11 miRNAs (miR-2944a-5p, miR-92b, miR-989, miR-275, miR-281-3p, miR-281-5p, miR-306, miR-263a-5p, miR-7, miR-309 and miR-305-3p) in *An. stephensi* whole body samples were shown to be significantly induced in blood fed mosquitoes at 42 h PBM, while miR-190-3p and miR-124 were down regulated 42 hours PBM (86). Another study reported that the expression of miR-989 was predominately expressed in the ovaries in *An. stephensi* and *Ae. aegypti* (79). It was also shown that miR-989 is highly expressed in the female *An. anthropophagus* mosquitoes but remains undetectable in male mosquitoes (85). Small scale expression profiling experiments of conserved miRNAs in the *Ae. aegypti* fat body (61) and whole body mosquitoes (72) has also displayed up regulation of many miRNAs after blood feeding.

The first functional analysis of a mosquito miRNA was performed in *Ae. aegypti* on the conserved miRNA, miR-275 (61). Expression profiling of miR-275 in the female mosquito fat body suggested regulation by 20E. Indeed, *in vitro* tissue culture of non-blood fed mosquito fat bodies incubated with 20E displayed an activated miR-275 response. Inactivation of miR-275 resulted in dramatic defects in blood digestion, excretion and, consequently, egg development in female mosquitoes. Upon 24 hours PBM, blood that had entered the midgut during blood feeding was regurgitated into the crop, an anterior portion of the digestive system normally used for housing nectar. Blood in the crop and residual blood in the posterior midgut remained undigested, and as a result ovary maturation was severely inhibited. The exact role of miR-275 in regulating digestive system processes and the identification of miR-275 targets contributing to this phenotype have not been identified. In *Ae. albopictus*, depletion of the mosquito-specific miRNA, miR-1891, results in a significant reduction in longevity and a marked reduction in fecundity (89), indicating a possible role in regulating blood meal associated events in the female mosquito. These studies raise a question concerning involvement of both conserved and mosquito-specific miRNAs in regulating blood meal activated functions in female mosquitoes.

1.7 MicroRNA regulation of mosquito-pathogen interactions

MicroRNA expression patterns in female mosquitoes also change in response to pathogen infection (78, 84, 86, 90-92), implying a function in mediating an immune response to invading pathogens. In *An. gambiae* female midguts infected with the malaria

model organism *Plasmodium berghei*, miR-34, miR-1174 and miR-1175 displayed down-regulation while miR-989 exhibited a significant up-regulation in expression (78). Whole body sRNA libraries of *Plasmodium vinckei petter*-infected verses non-infected *An. stephensi* mosquitoes 42 hours PBM indicated miR-124, miR-137, miR-1000 and miR-932 were significantly up-regulated in malaria-infected mosquitoes (86). In the same experiments, malaria-infected female mosquitoes displayed up-regulation of miR-1175-3p, miR-1174, miR-281-3p and miR-281-5p and down-regulation of 10 miRNAs (miR-285, miR-2944a-5p, miR-309, miR-210-3p, miR-1891, miR-981, miR-315-5p, miR-932, miR-124 and miR-7) 5 days PBM. Microarray abundance analysis of miRNAs in *An. gambiae* mosquitoes infected with the most deadly form of the malaria parasite *P. falciparum*, showed elevated abundance of miR-989 and miR-305 in infected midguts (90). Inhibition of miR-305 resulted in increased resistance to *P. falciparum* infection, while treatment with miR-305 mimic increased susceptibility to *P. falciparum* infection, suggesting miR-305 functions in regulating the mosquito immune response.

Studies regarding miRNA machinery activity further substantiates miRNA involvement in regulating the malaria infection (78, 93). Dcr-1 and Ago-1 knockdown in *P. berghei* susceptible mosquitoes resulted in a substantial increase in *Plasmodium* oocysts numbers (78). Additionally, polysome profiling of actively translated mRNAs in *An. gambiae* mosquitoes after infection with the most deadly form of the malaria parasite, *P. falciparum*, revealed enhanced associations of Dcr-1, Dcr-2 and Drosha with polysomes (93). These studies suggest that the miRNA machinery play a role in regulating mosquito response to infection by the malaria parasite.

In addition to parasitic infection, mosquito miRNAs have been shown to regulate viral infection. CHIKV infection specifically alters miRNA expression profiles in infected mosquitoes (84, 91, 94, 95). Differential expression analysis of miRNAs in *Ae. albopictus* C7/10 mosquitoes cells and *Cu. quinquefasciatus* mosquitoes infected with West Nile virus (WNV) displayed down-regulation of miR-989 and induction of miR-92 (84). Another study showed that in *Ae. albopictus* (Singh) cells, most of miRNAs were down-regulated upon CHIKV infection; however, miR-100, miR-283, miR-305-3p and miR-927 were significantly induced post infection, while miR-1000, miR-2b, miR-2c-3p and miR-190-5p were under expressed post infection (94). The mosquito-specific miR-2940 was shown to be down-regulated in C6/36 mosquito cells in response to WNV infection (95). This miRNA facilitates virus propagation by upregulating the host metalloprotease *m41 FtsH*, which is required for efficient WNV replication. Both depletion of miR-2940 or metalloprotease reduced WNV titer in C6/36 cells. More recently, miRNAs were detected in *Ae. aegypti* and *Ae. albopictus* saliva, 31 miRNAs that were previously unidentified and were designated as novel (91). The effect of CHIKV infection in miRNA expression in the saliva resulted in the up-regulation of 59 and 30 known miRNAs in *Ae. aegypti* and *Ae. albopictus*, respectively. Together these results indicate a functional importance of host miRNAs in mediating an immune response to CHIKV, and in regulating CHIKV dissemination and transmission.

Profound changes in miRNA expression occur in response to DENV infection (92). DENV2-exposed *Ae. aegypti* sRNA libraries have revealed modulation of 35 miRNAs in response to DENV-2 infection (92). Additionally, miR-375 was detected in

female *Ae. aegypti* mosquitoes PBM (72). Target sites complementary to miR-375 were identified and confirmed in the 5'-UTRs of the Toll immune pathway components Cactus and REL1. Female mosquitoes treated with mimic miR-375 molecules resulted in down-regulation of Cactus and up-regulation of REL1 transcripts. In addition, enhanced DENV infection was observed in *Ae. aegypti* Aag2 DENV-infected cells treated with miR-375 mimic possibly due to an up-regulation of Cactus which may block the expression of innate immune genes and antimicrobial peptides.

Mosquito miRNAs also have the potential to target the viral genome (96, 97). *Ae. albopictus* miR-252 is induced after DENV-2 infection and was shown to target DENV-2 DEV-2 E protein, a protein required for cell attachment and viral entry (96). This interaction between the host miRNA, miR-252, and the viral genome implies that miR-252 may act as a cellular antiviral regulator in *Ae. albopictus*. In another study, *Ae. albopictus* miR-281 was shown to be upregulated after DENV-2 infection (97). Inhibition of miR-281 in female mosquitoes led to a significant reduction in DENV-2 abundance. miR-281 was shown to potentially target the DENV-2 genomic 5' UTR SLA region, indicating an interaction between a host miRNA and the DENV genome, with DENV exploiting this host miRNAs for the benefit of its replication.

Endosymbionts have also been shown to interact with a mosquito host through the hijacking of mosquito miRNAs and miRNA machinery (65, 98). Successful introduction of the maternally inherited endosymbiotic bacteria *Wolbachia pipientis* into *Ae. aegypti* mosquitoes results in decreased lifespan, feeding success, and arbovirus replication. In *Ae. aegypti* mosquitoes infected with the *Wolbachia* strain wMelPop-CLA, 35 miRNAs

displayed differential expression in response to this endosymbiont (65). miR-12 was up-regulated in *Ae. aegypti* infected with wMelPop-CLA (65, 98). Inhibition of miR-12 in *Wolbachia*-infected Aag2 mosquito cells resulted in a reduction of *Wolbachia* density (98). Furthermore, miR-2940 was shown to be exclusively expressed in *Wolbachia*-infected mosquitoes and interacts with the 3' UTR of the *metalloprotease m41 ftsh* transcript, causing a stabilization of *metalloprotease m41 ftsh* transcripts. Both miR-2940 and *metalloprotease m41 ftsh* were shown to be crucial for *Wolbachia* replication. Another study identified that *Ae. aegypti* DNA methyltransferase gene, Dnmt2, is targeted by miR-2940 as well (99). Expression of Dnmt2 suppresses *Wolbachia* replication while enhancing DENV-2 replication in mosquito cells, suggesting that miR-2940 regulation of Dnmt2 plays an important role in the maintenance of *Wolbachia* infection and resistance of mosquito cells to DENV infection. These studies imply that mosquito endosymbionts hijack mosquito miRNAs to enhance endosymbiont colonization.

1.8 MicroRNA role in insecticide resistance in mosquitoes

Pyrethroids, such as deltamethrin, are the major class of insecticides used for mosquito control. Excessive use of insecticides has resulted in pyrethroid resistance in mosquito disease vectors, posing a major obstacle for mosquito control. Cytochrome P450 monooxygenase genes have been shown to be important in the resistance of mosquitoes to insecticides (100). In a comparison of miRNA expression in deltamethrin-resistant versus -sensitive *Cu. pipiens* 28 differentially expressed miRNAs were

identified between the two strains (101). One such miRNA, miR-71, was significantly down-regulated in female adults from the deltamethrin-resistant strain. Increased miR-71 resulted in enhanced mortality rate in deltamethrin-resistant mosquitoes. Furthermore, miR-71 was shown to participate in pyrethroid resistance through interactions with the 3' UTR of *cytochrome P450 325BG3 (CYP325BG3)*. In addition, miR-278-3p was shown to be up-regulated in the deltamethrin-susceptible strain of *Cu. pipiens* (101, 102). It was shown that miR-278-3p interacts with the 3'UTR of *CYP6AG11*, another cytochrome P450 gene (102). Mosquitoes treated with miR-278-3p mimic displayed increased susceptibility to deltamethrin, indicating a role in miRNAs regulating pyrethroid resistance.

1.9 Dissertation Objectives and Aims

The studies described above indicate the importance of miRNAs in regulating physiological processes important for the adult mosquito life stages. The discoveries of mosquito-specific miRNAs suggest functions common to most mosquitoes, such as blood feeding processes, vitellogenesis and mosquito-pathogen interactions. This dissertation highlights three important studies of miRNAs during the gonadotrophic cycle of the female mosquito, *Aedes aegypti*. Chapter II investigates the function of the conserved miRNA, miR-8, and its tissue specific role in the fat body of the female mosquito, *Ae. aegypti*. Chapter II further examines the physiologically relevant target of miR-8, *Secreted Wingless signaling interacting molecule (Swim)* and its role in regulating YPP secretion by the fat body. Chapters III and IV focus on the role of two lineage-specific

miRNAs in the midgut of the female mosquito. Chapter III investigates the function of the miR-1174/miR-1175 cluster of two important disease vectors, *Ae. aegypti* and *An. gambiae*, and describes the interaction between miR-1174 and its target, *Serine hydroxymethyltransferase (SHMT)* in regulating fluid excretion, blood digestion and, consequently, egg development. Chapter IV takes an alternative approach to identifying miRNA targets, in which putative miRNA binding sites are predicted in known genes important for blood digestive processes in *Ae. aegypti*. Chapter IV continues by characterizing the interaction between the juvenile hormone-regulated chemotypsin-like protease, *JHA15*, and the ecdysone regulated mosquito-specific miRNA, miR-1890. Together these chapters work to enhance our understanding of both conserved and lineage-specific miRNAs, and their role in regulating reproductive and blood-digestive processes in the female mosquito. These studies have the potential to influence the development of a new generation of mosquito control measures targeting the disease vectors ability to reproduce.

1.10 References

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CHAPTER II

MicroRNA-8 Targets the Wingless Signaling Pathway in the Female Mosquito Fat Body to Regulate Reproductive Processes

2.1 Abstract

Female mosquitoes require a blood meal for reproduction, providing the underlying mechanism for the spread of many important vector-borne diseases in humans. A deeper understanding of the molecular mechanisms linked to mosquito blood meal processes and reproductive events is of particular importance for devising innovative vector control strategies. We uncovered that the conserved miRNA, miR-8, is an essential regulator of mosquito reproductive events. Two strategies to inhibit miR-8 function *in vivo* were employed for functional characterization: systemic antagomir depletion and spatiotemporal inhibition utilizing the miRNA sponge transgenic method in combination with the Gal4/UAS system. Depletion of miR-8 in the female mosquito results in defects related to egg development and deposition. We used a multi-algorithm approach for miRNA target prediction in mosquito 3' untranslated regions, and experimentally verified *Secreted Wingless-interacting molecule (Swim)* as an authentic target of miR-8. Our findings demonstrate that miR-8 controls the activity of the long-range Wingless (Wg) signaling through regulation of *swim* expression in the female fat body. We discovered that the miR-8/Wg axis is critical for the proper secretion of lipophorin and vitellogenin by the fat body and subsequent accumulation of these yolk protein precursors by developing oocytes.

2.2 Introduction

Female hematophagous mosquitoes require a blood meal for reproduction, resulting in the transmission of many devastating vector-borne diseases in humans. Lack of vaccines, increasing drug resistance in pathogens, and insecticide resistance in vectors add to the urgency to explore alternative vector control strategies. Acquisition of blood initiates a cascade of events in several tissues in the female mosquito. The fat body, an adipose tissue analogous to the mammalian liver, plays a prominent role in energy metabolism, immunity and reproduction. The adult female mosquito fat body undergoes dynamic changes, before and after a blood meal, to accommodate ovarian development and other physiological necessities (1-3). The fundamental step in these events is vitellogenesis, in which the intake of blood induces the synthesis and secretion of yolk protein precursors (YPPs) in the fat body and subsequent accumulation in the developing oocytes (1, 4). Despite the continuing efforts to identify factors controlling mosquito reproduction, studies regarding the role the fat body plays during ovarian development have not revealed the entire governing complexity of mosquito reproductive biology.

The discovery of small non-coding RNAs has revolutionized our understanding of complex gene networks. One class of endogenous small RNA, known as microRNAs (miRNAs), plays key regulatory roles of gene expression at the post-transcriptional level (5). Animal miRNAs exert their action by base pairing of the miRNA “seed” to complementary sites within the 3' untranslated region (UTR) of mRNA, resulting in translational inhibition and mRNA decay (5, 6). While the function of miRNAs are a heavily studied topic in model organisms, such as the fruit fly *Drosophila melanogaster*,

information regarding the exact functional role of miRNAs in mosquito biology remains largely unknown (5, 7). However, recent functional studies of specific miRNAs have linked miRNA action in mosquitoes to reproductive events (7, 8). Studying miRNA function in the female mosquito is crucial in developing a full understanding of the regulatory processes of mosquito reproduction, and may pave the way towards the utilization of these small molecules as novel control approaches.

The miR-8/miR-200 family of miRNAs is one of the most widely studied miRNA families, and has emerged as important regulators in animal development and disease (5, 9, 10). Previously, we reported that miR-8 exhibits high levels of expression in the female mosquito fat body post-blood meal (PBM) (11), suggesting that miR-8 might have a role in the regulation of female mosquito adult stages. Here, we describe our study to functionally characterize miR-8 in the female mosquito fat body, and to establish an interaction between miR-8 and its target *Secreted Wingless-interacting molecule* (Swim). This study provides evidence that miR-8 acts on the Wingless (Wg) signaling pathway and functions in the fat body of the female mosquito affecting reproductive processes. miR-8 functions as a regulator of the *Ae. aegypti* homolog of *Drosophila Secreted Wingless-interacting molecule* (swim), thereby controlling the long-range Wg signaling activity. Thus, we identified the role of miR-8 and Wg/Swim in the secretory activity of the fat body in an adult female insect and elucidated their roles in reproduction.

2.3 Materials and Methods

Mosquito Rearing

The *Ae. aegypti* wild-type UGAL/Rockefeller strain and transgenic lines were reared at 27 °C and 80% humidity, as described previously (12). Blood feeding of adult mosquitoes was performed using White Leghorn chickens.

DNA Transformation Constructs

The Vg-Gal4 driver and pBac[3xP3-DsRed] transformation vectors were produced as previously described (13). The germ-line transformation vectors carrying the UAS-miR8-SP and UAS-Scr-SP were constructed as described below. *Drosophila* miR-8-SP and non-specific control (UAS-Scr-SP) cassettes in a pUAST-EGFP vector were provided by Dr. Tudor Fulga (Department of Molecular Medicine, University of Oxford) (12). The 4.0kb UAS-miR8-SP BamHI fragment was subcloned into the pBac[3xP3-DsRed] transformation vector using the BglIII unique cloning site.

Germ-line Transformation

The germ-line transformation for the Vg-Gal4 driver line was performed as described previously (13, 14). PiggyBac mediated germ-line transformation for the UAS-miR8-SP and UAS-Scr-SP responder lines were carried out by the University of Maryland Institute for Bioscience and Biotechnology Research Insect Transformation Facility. The Vg-Gal4/UAS-miR8-SP and Vg-Gal4/UAS-Scr-SP hybrid mosquito lines were established as previously described (13, 14).

Antagomir and dsRNA Treatments

Antagomirs were obtained from Dharmacon using the RNA module for custom single-stranded RNA synthesis available at <http://www.dharmacon.com/rna/rna.aspx>. Antagomir to miR-8 was 5' mG.*.mA.*.mC.mA.mU.mC.mU.mU.mU.mA.mC.mC.mU.mG.mA.mC.mA.mG.mU.mA.*.mU.*.mU.*.mA.*.mA. -Chl 3'. "*" is a Phosphorothioate backbone instead of a PO backbone. "m" is an OCH₃ group on the 2' end of the base instead of an OH group. A 3' cholesterol (Chl) group was added to each RNA oligo for potency reasons. Antagomirs were constructed as previously described (8, 11). Mosquitoes were CO₂ anesthetized 12 h post eclosion (PE) and microinjected into the thorax at a dose of 200 μ M in a volume of 0.25 μ L (50 pmol). Mosquitoes were allowed to recover for 3 to 4 days before blood feeding.

Double-stranded RNA (dsRNA) was produced as described previously (12). In brief, dsRNA was synthesized using the MEGAscript kit (Ambion). At 12 h PE, female mosquitoes were microinjected into the thorax with 0.5 μ g (0.25 μ L of 2 μ g/ μ L) dsRNA. For rescue experiments, mosquitoes were co-injected with 0.25 μ L of an antagomir/dsRNA mixture with a final concentration of 200 μ M antagomir and 2 μ g/ μ L dsRNA.

Total RNA Extraction and Real-Time PCR

Total RNA was extracted from tissue using the TRIzol method (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's protocol. miRNA

expression was measured as previously described (8, 11). In brief, cDNAs for miRNAs was produced using miScript II RT Kit (Qiagen) and qRT-PCR was performed using the miScript SYBR Green PCR kit (Qiagen) according to manufacturer's protocol. For mRNA, cDNAs were synthesized from 2 µg total RNA SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed using the QuantiFast SYBR® Green PCR Kit (Qiagen). Quantitative measurements were performed in triplicate and relative expression (RE) was measured as $RE = 2^{-\Delta\Delta Ct}$. Normalization was performed against the housekeeping genes (HKGs), S7 ribosomal protein (RPS7) and actin, using the geometric average of the HKGs Ct values. Percent relative expression (% RE) was calculated against wild-type (UGAL or non-injected) control.

Immunoblot

Protein analysis of Vg and Lp was done as described previously (12). Tissues from blood-fed female mosquitoes were homogenized in lysis buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1× phosphatase inhibitor from Sigma cat # P2850, and 1× protease inhibitor from Sigma cat # P8340). 10 µg protein was boiled in LDS (4X) NuPage sample buffer (Invitrogen) with 10X sample reducing agent (Invitrogen) and run on 4-12% Tris-Glycine gels (Invitrogen) before being transferred to PVDF membranes. For detection of Vg, Vg monoclonal antibodies (15) was used at 1:5000 dilution; followed by the secondary anti-mouse-HRP (Sigma) at 1:2000 dilution. For detection of Lp, apolipoprotein-I (apoLp-I) polyclonal antibody (16) was used at 1:10000 dilution followed by the secondary anti-

rabbit-HRP (Roche) a 1:10000 dilution. For detection of Actin, beta-actin monoclonal antibody (Sigma) was used at 1:5000 dilution followed by the secondary anti-mouse-HRP (Sigma) at 1:2000 dilution.

in vitro Fat Body Culture

Fat bodies were dissected from mosquitoes 4 days after microinjection in Aedes physiological saline (APS) buffer and were incubated in a complete culture medium supplemented with amino acids and 20-hydroxyecdysone (concentration used was 1 μ M; Sigma) for 6 h, as described previously (12). An aliquot of complete culture medium was removed and trichloroacetic acid (TCA)-precipitated using ProteoPrep Protein Precipitation Kit (Sigma). Precipitants were dissolved in 1X Laemmli Sample Buffer (Sigma). Total protein was quantified using the Bio-Rad Protein Assay. 2 μ g of protein was used for immunoblot as described above.

Nile Red and DAPI Staining

Ovaries were harvested from mosquitoes 24 h post blood meal (PBM) and fixed in 4% (vol/vol) paraformaldehyde at room temperature for 1 h with shaking, as previously described (8). Fixed tissues were incubated in a solution (20% glycerol in PBS, with a 1:10,000 dilution of 10% Nile Red, from Sigma, in DMSO) for 2 h at room temperature. Tissue was mounted using ProLong® Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) and visualized on Leica SP5 confocal microscope using the 20x objective lens.

Computational Target Prediction

Computational target prediction was performed as previously described (8). The readily available miRNA target prediction programs that were used are: TargetScan, PITA, miRANDA, and RNAhybrid. In addition, an “in-house” miRNA target prediction program was also utilized. 3' UTRs from the *Ae. aegypti* AaegL1.3 and *An. gambiae* Agam3.7 genebuilds were used.

Cell Culture and Luciferase Assay

In vitro target validation was performed as previously described (8). *Drosophila* Schneider 2 (S2) cells (Invitrogen) were kept at 28°C in Schneider's *Drosophila* medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies) and 1× Antibiotic-Antimycotic (Gibco, Life Technologies). Luciferase constructs were made by inserting the *Ae. aegypti* miR-8 putative target 3' UTRs into the multiple cloning region located downstream of the Renilla translational stop codon within the psiCheck-2 vector (Promega). 100ng of psiCheck-2 reporters and synthetic aae-miR-8 miScript miRNA Mimic (Qiagen) or AllStars Negative Control siRNA (Qiagen) at a final concentration of 100nm were co-transfected into *Drosophila* S2 cells using Attractene Transfection Reagent (Qiagen). A no mimic treatment was also performed. Dual Luciferase Reporter assay was completed 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase was used for normalization of Renilla luciferase expression. Treatments were made in triplicate, and transfections were repeated three times.

2.4 Results

miR-8 is enriched in the female mosquito fat body post-blood feeding

To produce a thorough time course expression analysis of mature miR-8 in the adult female mosquito fat body, we measured mature miR-8 relative expression levels by quantitative real time (qRT) PCR analysis. Mature miR-8 levels were low in all tissues PE; however, expression levels substantially increased in the female mosquito fat body post-blood meal (PBM) (Fig. S2.1A). We monitored mature miR-8 abundance in the fat body using eight time points collected over the first reproductive cycle. We obtained total RNA samples from female mosquito fat bodies at 0-6, 24, 48 and 72 h PE, and 12, 24, 36, 48 and 60 h PBM. In this tissue, mature miR-8 was significantly up-regulated by 12 h PBM, reaching its peak by 48 h PBM and declining by 60 h PBM (Fig. S2.1B). These results suggest that the high expression of miR-8 in the female mosquito fat body may play an important role in tuning events associated with the adult life stage of the female mosquito.

Systemic miR-8 depletion results in abnormal ovarian development

In order to assess the function of miR-8 in the adult female mosquito, we achieved a knockdown using a miRNA specific antisense oligonucleotide (antagomir). We designed an antagomir consisting of the reverse complement of miR-8 (miR-8Ant), as well as a randomly scrambled “missense” antagomir (MsAnt) for a control. Female mosquitoes were microinjected at a dose of 50 pmol per mosquito of the miR-8Ant or MsAnt at 12 h PE. To evaluate efficiency of the miR-8 depletion, we monitored

endogenous levels of mature miR-8 by means of qRT-PCR. Sequence specific antagomirs designed to target miR-8 successfully depletes mature miR-8 in the female mosquito fat body by 24 h PBM when compared to the MsAnt and non-injected controls (Fig. S2.1C). The miR-8Ant had no effect on the relative expression levels of other miRNAs, such as mature miR-275 (Fig. S2.1D).

Female mosquitoes treated with the miR-8Ant were screened for phenotypic manifestations. First, we evaluated the state of ovarian development in mosquitoes at 24 h PBM. Blood fed females treated with the miR-8Ant failed to fully develop vitellogenic ovaries during the first gonadotrophic cycle compared to the MsAnt and non-injected controls (Fig. 2.1A). The degree to which females treated with the miR-8Ant displayed this phenotype varied considerably across individuals. Ovarian follicle growth was drastically inhibited in miR-8 depleted females compared to control mosquitoes, with an average primary follicle length of 154.7 μm (Fig. 2.1B; Fig. S2.2C). Ovaries from MsAnt-treated females were similar to those in non-injected female mosquitoes at 24 h PBM, with primary follicles reaching 200 – 210 μm in length on average (Fig. 1B; Fig S2.2A-B). In addition, miR-8Ant-treated females displayed reduce fecundity, laying a significantly reduced number of eggs per mosquito as compared to controls, with an average of 36.3 eggs per mosquito (Fig. 2.1C). MsAnt-treated females laid a similar number of eggs as non-injected female mosquitoes, with 92.8 and 91.63 eggs per female on average, respectively (Fig. 2.1C). Previtellogenic ovary development (Fig. S2.2D), host seeking behavior, and blood digestion were unaffected in miR-8Ant-treated females,

and displayed no other adverse phenotypes when compared to MsAnt-treated and non-injected controls.

Spatiotemporal inhibition of miR-8 reveals function specific to the female mosquito fat body

Antagomir mediated miRNA depletion is based on microinjections, creating systemic effects without clear indication of tissue specificity. Therefore, we asked if the miR-8Ant phenotype is a result of miR-8 reduction in the fat body or another tissue. To answer this, we achieved a spatiotemporal inhibition of miR-8 using the miRNA sponge (miR-SP) transgenic method. Due to the high conservation of miR-8, a *Drosophila* UAS-miR8-SP cassette was utilized (17) in combination with the previously established *Aedes* Vg-Gal4/UAS system (13). The fat body-specific system utilizes the Vg gene promoter to drive transgene expression (13), permitting precise spatiotemporal genetic testing in vivo of miR-8 functions in the female mosquito fat body PBM (Fig. S2.3A).

The UAS-miR8-SP and control “scramble” (UAS-Scr-SP) cassettes were subcloned into a piggyBac-based responder construct. Responder plasmids contained five repeat concatemers of the upstream activating sequence (UAS), with a TATA box and hsp70 minimal promoter linked to the miR-SP cassette, and a SV40 polyadenylation sequence (Fig. S2.3B). Responder transposons also included a dsRed selectable marker gene under the 3xP3 eye-specific promoter (Fig. S2.3B). Responder constructs were sent to the Insect Transformation Facility at the University of Maryland for injection into *Ae. aegypti* Orlando strain. Three independent UAS-miR8-SP responder lines and three

independent UAS-Scr-SP were generated. Responder lines were selected based on a strong expression of eye-specific dsRed selectable marker. Genomic PCR analysis was conducted to confirm a stable incorporation and the integrity of UAS-miR8-SP and UAS-Scr-SP constructs into the *Ae. aegypti* genome (Fig. S2.3C). Vg-Gal4/UAS-miR8-SP and Vg-Gal4/UAS-Scr-SP hybrid transgenic lines were produced by crossing homozygous responder lines with homozygous Vg-Gal4 driver. Genomic PCR analysis confirmed presence of both transgenes in hybrid lines (Fig. S2.3D). Hybrid transgenic lines were selected by simultaneous presence of both EGFP and dsRed eye-specific markers (Fig. S2.4).

For further analyses, we selected two Vg-Gal4/UAS-miR8-SP and two Vg-Gal4/UAS-Scr-SP hybrid lines based on a strong expression of the eye-specific marker genes (Fig. S2.4). We analyzed mature miR-8 levels by means of qRT-PCR in the Vg-Gal4/UAS-miR8-SP, Vg-Gal4/UAS-Scr-SP, Vg-Gal4 driver line and wild-type (WT) females. Mature miR-8 remained unaffected in the Vg-Gal4/UAS-Scr-SP hybrid lines, Vg-Gal4 driver and WT females, while the Vg-Gal4/UAS-miR8-SP hybrid lines displayed a reduction of mature miR-8 expression 24 h PBM (Fig. 2.1D). The Vg-Gal4/UAS-miR8-SP, Vg-Gal4/UAS-Scr-SP, Vg-Gal4 driver and WT females were subjected to phenotypic analysis. There were no detectable negative effects on mosquito development, fecundity, viability or sex ratio in the Vg-Gal4/UAS-Scr-SP hybrid lines, Vg-Gal4 driver or WT females.

Similar to miR-8Ant-treated females, blood fed Vg-Gal4/UAS-miR8-SP females displayed an inhibition of ovarian development and reduced fecundity. Ovaries from the

Vg-Gal4/UAS-Scr-SP hybrid lines and Vg-Gal4 driver were similar to those in WT female mosquitoes at 24 h PBM with primary follicles reaching 213.15 μm in length on average (Fig. 2.1E; Fig. S2.5A-C). Ovarian follicle growth was considerably reduced in the Vg-Gal4/UAS-miR8-SP hybrid lines at 24 h PBM compared to control mosquitoes, with an average primary follicle size of 192.4 μm in length (Fig. 2.1E; Fig. S2.5D). Vg-Gal4/UAS-miR8-SP hybrid follicles were very heterogeneous in size compared to controls, ranging from 88 – 240 μm in length. Additionally, blood fed Vg-Gal4/UAS-miR8-SP hybrid females laid a significantly reduced number of eggs per mosquito as compared to controls, with an average of 44.1 eggs laid per mosquito (Fig. 2.1F). Additional striking phenotypes were also detected in the Vg-Gal4/UAS-miR8-SP hybrid lines. When female ovaries were observed at 5 days PBM, Vg-Gal4/UAS-miR8-SP hybrid lines continued to retain eggs, which began to undergo melanization within the ovary - a process that is normally observed after oviposition (Fig. S2.5E-F). Previtellogenic ovary development (Fig. S2.5G), host seeking behavior and blood digestion were unaffected in the Vg-Gal4/UAS-miR8-SP hybrid females, and displayed no other adverse phenotypes when compared to controls. Together these results indicate that miR-8 action in the female mosquito fat body, in large measure, is responsible for the miR-8 depletion phenotype in the ovary.

Computational and in vitro analysis indicates miR-8 targets Secreted Wingless-interacting molecule

To identify the putative gene targets of miR-8, we took an in-silico approach, as previously described (8), using five different target prediction programs – TargetScan (18), PITA (19), miRanda (20), RNAhybrid (21) and a program developed in-house (8). To assess for conservation criteria, we predicted miR-8 targets using *Ae. aegypti* and *Anopheles gambiae* 3' UTRs. We extracted the 3' UTRs from the *Ae. aegypti* (AaegL1.3) and *An. gambiae* (AgamP3.7) genome gene builds, for use within each target prediction program. Overlapping predicted targets between each program was assessed, and a list of top candidate targets was produced based on the predictions by multiple algorithms and conservation (Table 2.1). The *Drosophila* ortholog of AAEL001232, *Secreted Wingless-interacting molecule* (*swim*), has been recently identified to function in the Wingless (Wg) signaling pathway to maintain the Wg ligand stability for long-range Wg signaling (22). Additionally, *Drosophila* miR-8 is known to target many players in the Wg signaling pathway (23). Due to this knowledge and conservation of the miR-8 binding site in *An. gambiae* (Fig. 2.2A), we chose AAEL001232 (*swim*) for further analysis.

Following computational prediction, we assessed the 3' UTR of *swim* for its response to miR-8 *in vitro*. The *swim* 3' UTR was cloned downstream of the Renilla translational stop codon within the psiCheck-2 vector to generate 3' UTR-fused luciferase reporters. When transfected into *Drosophila* Schneider 2 (S2) cells along with miR-8 mimic, the luciferase reporter containing the full-length aaeSwim 3' UTR yielded 69.75% luciferase activity compared to the negative control mimic and no mimic control samples

(Fig. 2.2B). Therefore, *swim* was validated as a potential target of miR-8 *in vitro* by means of Dual Luciferase Reporter Assay in S2 cells. The luciferase reporter assay was optimized using a known *Drosophila* miR-8 target, dmUSH (Fig. S2.6A).

miR-8 regulated Secreted Wingless-interacting molecule in vivo

Next, we monitored *swim* transcript levels in the fat body over the first reproductive cycle. We obtained total RNA samples from female mosquito fat bodies at 72 h PE, and 12, 24, 36 and 48 h PBM. Expression of *swim* declines early PBM, increasing in expression by 24 h PBM and again declining by 36 h PBM – corresponding to the highest levels of miR-8 expression (Fig. S2.6B). To evaluate *swim* as an authentic miR-8 target *in vivo*, we measured *swim* transcript levels in the fat body of miR-8Ant, Vg-Gal4/UAS-miR8-SP and control females, via qRT-PCR. This revealed that *swim* is significantly enriched in the miR-8 depleted female mosquito fat body at 24 h PBM compared to control females (Fig. 2.2C; Fig. S2.6C). In *Drosophila*, overexpression of *swim* interferes with long range Wg signaling in fly imaginal discs (22). We assayed the level of the long range Wg signaling response gene *distal-less* (*dll*) and the short range Wg signaling response gene *senseless* (*sens*) in the fat body of miR-8Ant, Vg-Gal4/UAS-miR8-SP and control female mosquitoes. Fat body *dll* expression was significantly lower in the miR-8 depleted fat bodies compared to control females (Fig 2.2D; S2.6D). Short range Wg signaling remained largely unaffected in the fat body of the miR-8 depleted female mosquitoes (Fig 2.2E; Fig S2.6E). Expression of *sens* and *dll* were unaffected in the mosquito ovary of the miR-8 depleted females compared to controls (Fig S2.6F, G).

Together, this suggests that miR-8 depletion in female mosquitoes results in an impairment of long range Wg signaling in the fat body.

Next, we conducted phenotypic rescue experiments through *swim* RNA Interference (RNAi) in miR-8 depleted female mosquitoes. It was expected that the RNAi mediated knockdown of the physiologically relevant target of miR-8 would alleviate the adverse phenotypes caused by miR-8 depletion. Double-stranded RNA (dsRNA) designed to target *swim* transcripts successfully depletes *swim* transcript levels in female mosquitoes (Fig. 2.3A). We co-injected 50 pmol of miR-8Ant or MsAnt and 0.5 ug of Swim or Luc dsRNA into each female mosquito at 12 h PE. The inhibited ovary development and egg deposition phenotypes were not observed in the miR-8Ant/dsSwim-treated mosquitoes at 24 h PBM, while females treated with the miR-8Ant and Luciferase dsRNA control displayed inhibited ovary development and reduced egg deposition characteristic of miR-8Ant-treated mosquitoes (Fig. S2.6H, I). Additionally, the inhibited ovary development and egg deposition phenotypes were not observed in the MsAnt/dsSwim-treated and non-injected control mosquitoes at 24 h PBM (Fig. S2.6H, I). Likewise, injection of Swim dsRNA into Vg-Gal4/UAS-miR8-SP mosquitoes alleviated the inhibited egg deposition phenotypes observed in Vg-Gal4/UAS-miR8-SP females (Fig. 2.3B). Hence, Swim RNAi dramatically restored proper ovarian development in miR-8 depleted female mosquitoes, suggesting that *swim* is an authentic target of miR-8 *in vivo*.

For further *in vivo* analysis, we determined whether Swim RNAi would restore dll expression to normal levels in the miR-8Ant and Vg-Gal4/UAS-miR-8-SP female

mosquito fat bodies. While miR-8Ant/dsLuc-treated mosquitoes displayed the characteristic decrease in *dll* expression observed in miR-8 depleted female mosquitoes, miR-8Ant/dsSwim displayed restored levels of *dll* expression similar to that of control treatments (Fig. S2.6J). Likewise, injection of Swim dsRNA into Vg-Gal4/UAS-miR8-SP mosquitoes restored *dll* expression similar to control mosquitoes (Fig. 2.3C). Together, these results suggest that elevated levels of the *swim* transcript in miR-8Ant-treated and Vg-Gal4/UAS-miR8-SP female mosquitoes inhibit ovary development PBM and dramatically reduces fecundity possibly due to a disruption of long range Wg signaling.

To further investigate that Wg signaling is functioning in the female mosquito fat body, we measured relative expression levels of several putative Wg signaling pathway components from female mosquito fat bodies 72 h PE and 24 h PBM via qRT-PCR. Comparison of the 72 h PE and 24 h PBM samples revealed that transcript levels of Wg signaling pathway receptors, Frizzled-1 and Frizzled-2, and a downstream component of the Wg signaling pathway, Armadillo, displayed significant up-regulation in the female mosquito fat body 24 h PBM (Fig. S2.7A-D). Likewise, the expression of several Wg protein precursors tested also increased PBM (Fig. S2.7E-J). These results suggest that Wg signaling components are indeed present in the female mosquito fat body PBM and further supports our finding that Wg signaling plays a role in the female mosquito fat body.

miR-8 depletion results in impaired YPP secretion by the fat body and lipid accumulation in developing oocytes

Overexpression of Wg in the *Drosophila* fat body results in lethality at the pupal stage and a reduction of fat body mass in third instar larvae, demonstrating the role of Wg signaling in the fat body for fat regulation (24). Likewise, activation of the Wnt pathway in adipose tissue decreases fat mass in mammals (25, 26). Together with our findings, this may suggest that improper Wg signaling, such as disruption of miR-8 expression, may interfere with normal fat body functions – including the synthesis and secretion of YPPs by the female mosquito fat body. To test this hypothesis, Vitellogenin (Vg) and Lipophorin (Lp) transcript levels were measured at 24 h PBM in the female fat body via qRT-PCR analysis. Treatment with the miR-8Ant did not affect the expression of Vg (Fig. S2.8A) or Lp (Fig. S2.8B) compared to MsAnt-treated and non-injected controls. Western blot analysis of Vg and Lp proteins revealed no significant change in protein levels in miR-8Ant female fat bodies compared to controls (Fig. 2.4A), indicating that miR-8 is not needed for YPP gene expression in the fat body. However, treatment with the miR-8Ant resulted in a drastic reduction of YPP protein levels in the ovary compared to MsAnt-treated and non-injected controls (Fig. 2.4B), suggesting a disruption of secretion and/or uptake of YPP into miR-8 depleted female ovaries. To test this, we performed an in vitro fat body culture, in which fat bodies were dissected from miR-8Ant or control-treated females 4 days after microinjection and incubated in a complete culture medium supplemented with amino acids (AA) and 20-hydroxyecdysone (20E). Culture medium was harvested to test secreted proteins by the fat body. Treatment with the miR-

8Ant resulted in a drastic reduction of YPP protein levels in the culture media compared to MsAnt-treated and non-injected controls (Fig. 2.4C) with equal amounts of total protein (S2.8C), verifying a disruption of YPP secretion by the fat body. Nuclear staining (DAPI) of miR-8Ant-treated female ovaries revealed small primary follicles and large nurse cells (Fig. S2.8D) compared to MsAnt and non-injected controls (Fig. S2.8E, F), characteristic of impaired ovarian development. Lipids that accumulate in developing oocytes originate in the fat body and are transported to the ovaries by Lp and Vg (23). Depletion of Lp in *Drosophila* imaginal discs causes a reduction of transcriptional activity of long-range Wg target genes (24), suggesting a link between Lp and the Wg pathway. Nile Red staining of lipids in the miR-8Ant-treated mosquito ovaries depicted a drastic reduction in lipid content (Fig. S2.8G) compared to controls (Fig. S2.8H, I). Together these results suggest that miR-8/Wg axis is important for secretion of YPPs and lipid accumulation into the developing oocyte.

2.5 Discussion

Our results show that depletion of the conserved miRNA, miR-8, in the female mosquito fat body PBM results in severe defects linked to ovary development and egg deposition. Using the Gal4-UAS system in combination with a miR-8 sponge in *Ae. aegypti*, this study represents a significant step towards defining regulatory roles of miRNAs in a tissue specific manner in mosquito disease vectors. Our work illustrates that miR-8 functions as a regulator of reproductive events in the female mosquito fat body through fine-tuning the expression of *Ae. aegypti* homolog to *Drosophila Secreted*

Wingless-interacting molecule (*swim*) thereby regulating the activity of long-range Wg signaling. Systemic antagomir and fat body specific depletion of miR-8 resulted in dramatic impairment of ovarian development and egg deposition: females displayed ovaries with drastically smaller primary follicles and fail to properly deposit eggs, suggesting impairment in signaling between the fat body and ovary.

A multi-algorithm approach for miRNA target prediction was used to identify the physiologically relevant miR-8 target contributing to the miR-8 depletion phenotypes. We identified *swim* as a direct target of miR-8 *in vitro* and *in vivo*. Dual Luciferase Reporter assay using a luciferase reporter vector containing *Ae. aegypti swim* 3' UTR co-transfected along with miR-8 mimic resulted in a decrease in *Renilla* luciferase activity *in vitro*, while Swim RNAi rescue experiments recovered the miR-8 depletion phenotype *in vivo*. These results further confirmed *swim* as an authentic miR-8 target in mosquitoes. *Drosophila* and *Ae. aegypti* Swim contain a Somatomedin B-like domain, implicating functions in protein–extracellular matrix interactions (2, 22). Likewise, the hydrophobic Wg morphogen has been shown to diffuse through the extracellular space and regulate long-range targets by associating with solubilizing molecules such as Swim (22). In *Drosophila*, overexpression of *swim* in imaginal discs results in a disruption of Wg signaling and *in vitro* analysis indicated that increased concentrations of Swim outcompete the Wg receptor access to the Wg ligand (22). Indeed, depletion of miR-8 in the female mosquito fat body results in increased expression of *swim* transcript level and subsequent reduction in long-range Wg signaling activity in the fat body of the female mosquito. Rescue experiments also restored long range Wg signaling in the female

mosquito fat body. The function of Wg signaling in the fat body of a reproducing female insect had not been uncovered until now. However, it has been elucidated that Wg signaling plays a role in regulating follicle stem cells in the *Drosophila* ovary (27, 28). More recently, it was shown that muscle derived Wg controls fat mass within the fat body in *Drosophila*, suggesting that secreted Wg may transmit to and act on distant organs (24). However, systemic and fat body specific depletion of miR-8 in the female mosquito did not appear to disrupt long range Wg signaling in the ovary. Taken together these results indicate that miR-8 regulates long range Wg signaling through the *Ae. aegypti* Swim molecule in the fat body of the female mosquito, and consequently affects ovarian development and egg deposition.

In *Drosophila*, overexpression of Wg in the fat body results in lethality at the pupal stage and decreased fat body mass in third instar larvae, demonstrating the role of Wg signaling in the fat body for fat regulation (24). Likewise, activation of the Wnt pathway in adipose tissue decreases fat mass in mammals (25, 26, 29). Together with our findings, this may suggest that a distortion in proper Wg signaling may interfere with normal fat body functions, having consequences on mosquito reproductive processes. Swim and miR-8 are likely to function in this process to fine tune Wg signaling in the fat body, allowing proper fat body functions. The drastically smaller primary ovarian follicles in the miR-8 depleted females suggested that YPP synthesis, secretion and/or uptake were compromised. However, there was no significant difference in mRNA and protein expression of the two important YPP genes, Vg and Lp, in miR-8 depleted female mosquitoes. Instead, we identified a disruption of YPP secretion by the fat body. Swim

and miR-8 are likely to fine tune Wg signaling in the fat body, allowing proper YPP secretion – leading to normal ovarian development (Fig. 2.5).

In conclusion, our work has established a fundamental role for miR-8 in the female mosquito fat body. miR-8 target investigation has revealed an intriguing role for the Wg signaling pathway in the female mosquito fat body and reproduction. Further investigation of the Wg signaling pathway in the adult mosquito fat body is warranted in order to determine the exact function of fat body Wg and its role in the regulation of the YPP secretory pathway. This investigation has opened a new avenue towards understanding signaling pathways that are important for fat body function in adult insects during reproduction.

2.6 Acknowledgments

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Figure 2.1 Caption

Systemic and tissue specific depletion of miR-8 results in decreased follicle size and dramatically reduced egg number. (A) Female mosquito ovaries 24 h post blood meal (PBM). Ovaries were visualized using the Leica M165FC stereo microscope. Scale bar is 1mm. (B) Average follicle size of miR-8Ant-treated, MsAnt-treated and non-injected (non-inj) mosquitoes 24 h PBM. (C) Egg numbers per female mosquito for miR-8Ant-treated, MsAnt-treated and non-injected mosquitoes. (D) Mature miR-8 levels are decreased in the Vg-Gal4/UAS-miR8-SP (DA55/M3-2; DA55/M4-2) female mosquitoes when compared to the controls. (E) Average follicle size of Vg-Gal4/UAS-miR8-SP (DA55/M3-2; DA55/M4-2) miR-8 depleted females compared to Vg-Gal4/UAS-Scr-SP (DA55/M3-1; DA55/F5-1), Vg-Gal4 (DA55) driver and wild-type controls 24 h PBM. Data represents the average (F) Egg number per female mosquito for Vg-Gal4/UAS-miR8-SP (DA55/M3-2; DA55/M4-2) females compared to Vg-Gal4/UAS-Scr-SP (DA55/M3-1; DA55/F5-1), Vg-Gal4 (DA55) driver and wild-type controls. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.1

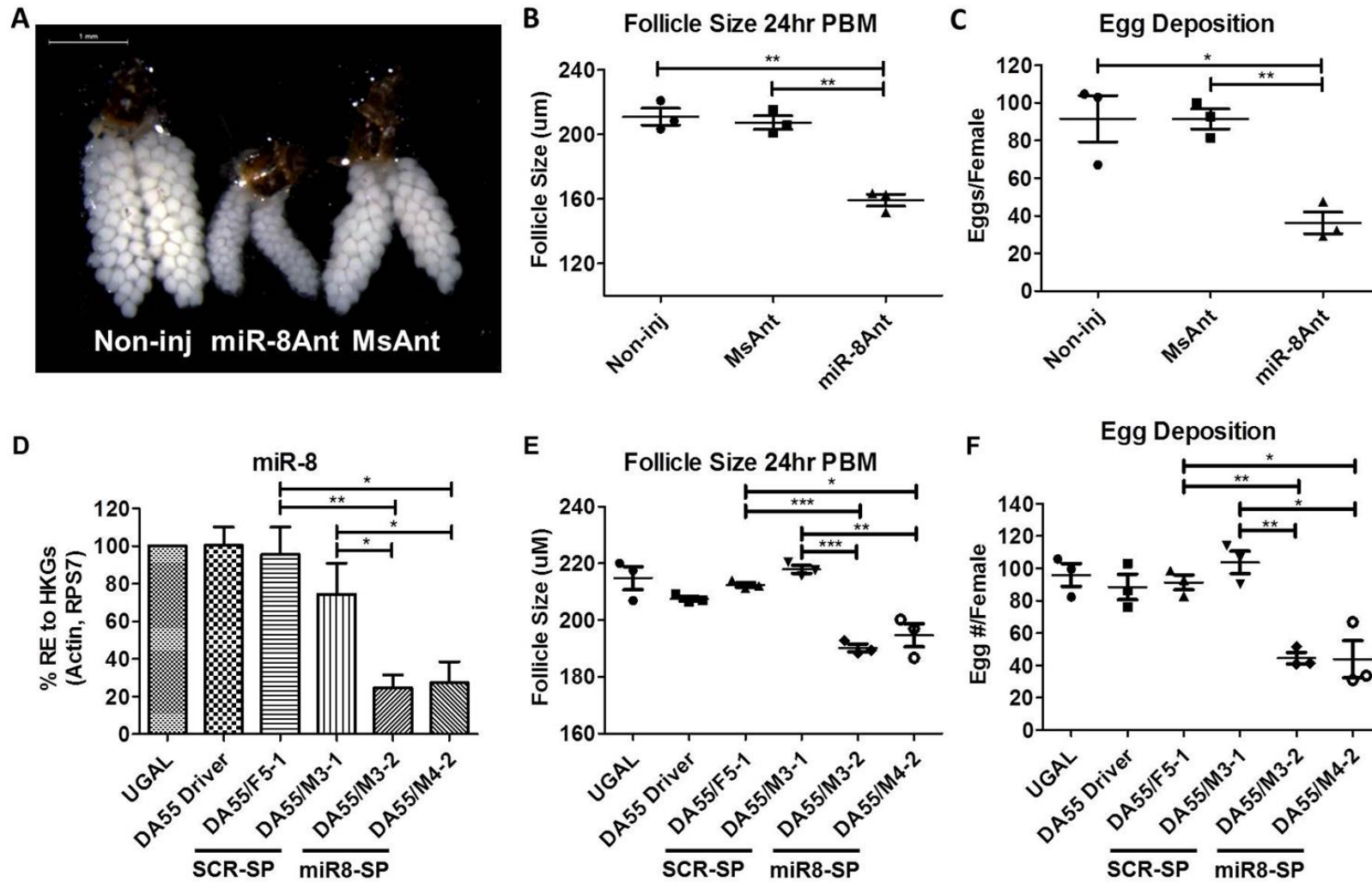


Figure 2.2 Caption

SWIM (AAEL001232) is a direct target of miR-8. (A) Putative miR-8 binding site within the AAEL001232 and AGAP007684 3'UTRs. (B) Dual Luciferase Reporter Assay for AAEL001232 (Secreted Wingless interacting molecule; Swim). Data represents the percent activity (Δ Fold Activity * 100) average $\pm$ SEM of triplicate samples. Percentages shown. (C) AAEL001232 expression increases in the Vg-Gal4/UAS-miR8-SP (DA55/M4-2) female mosquitoes compared to controls (WT, DA55, DA55/M3-1). (D) Long range Wingless signaling is reduced in the Vg-Gal4/UAS-miR8-SP (DA55/M4-2) female mosquitoes compared to controls (WT, DA55, DA55/M3-1). (E) Short range wingless signaling is unaffected in the Vg-Gal4/UAS-miR8-SP (DA55/M4-2) female mosquitoes compared to controls (WT, DA55, DA55/M3-1). Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.2

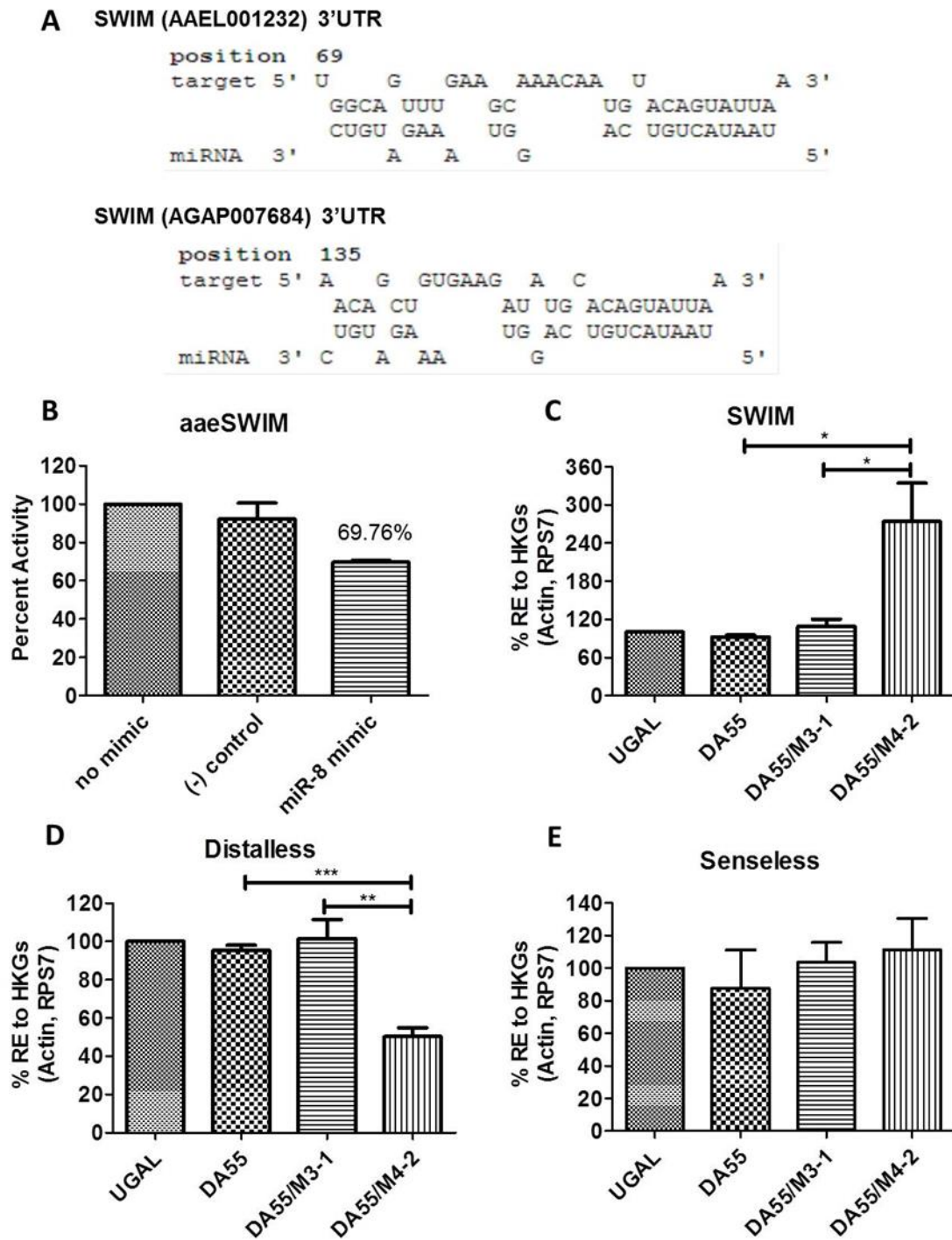


Figure 2.3 Caption

AAEL001232 RNAi rescues miR-8 depletion phenotypes. (A) Female mosquitoes were injected with 0.5 µg AAEL001232 (Secreted Wingless interacting molecule; Swim) or Luciferase dsRNA. Swim RNAi successfully knocks down *swim* transcripts *in vivo*. (B) Swim RNAi restores egg deposition phenotype of Vg-Gal4/UAS-miR8-SP (DA55/M4-2) mosquitoes. (C) Swim RNAi restores long-range Wingless signaling inhibition in Vg-Gal4/UAS-miR8-SP (DA55/M4-2) mosquitoes. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.3

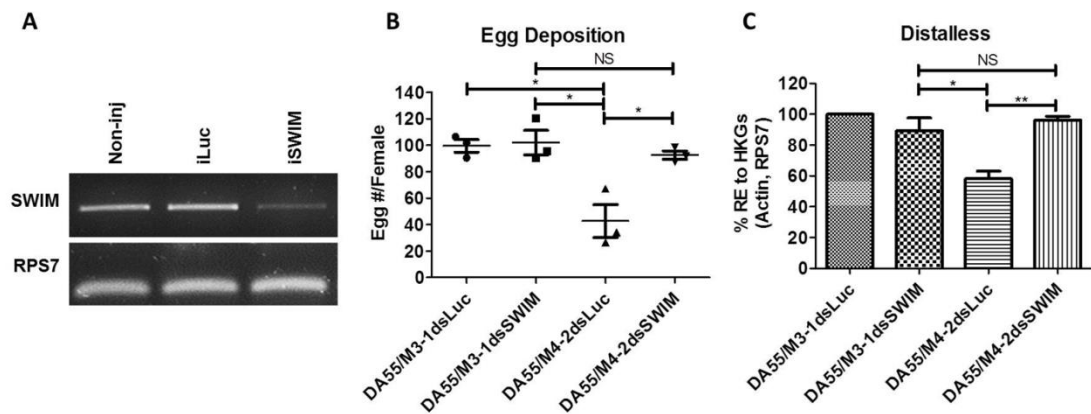


Figure 2.4 Caption

miR-8 depleted females display a disruption in yolk protein precursor secretion and lipid accumulation. (A) Western blot analyses utilizing antibodies against Vitellogenin (Vg) and Lipophorin (Lp) in miR-8Ant-treated, MsAnt-treated and non-injected (non-inj) female fat bodies. Beta-actin was used as a loading control (B) Western blot analyses utilizing antibodies against Vg and Lp in miR-8Ant-treated, MsAnt-treated and non-injected female ovaries. Beta-actin was used as a loading control (C) Western blot analyses of proteins secreted during *in vitro* fat body culture utilizing antibodies against Vg and Lp from miR-8Ant-treated, MsAnt-treated and non-injected female fat bodies.

Figure 2.4

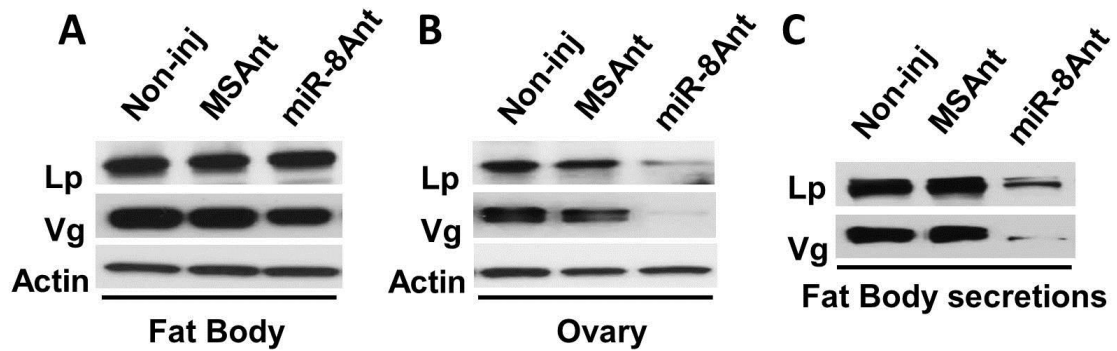
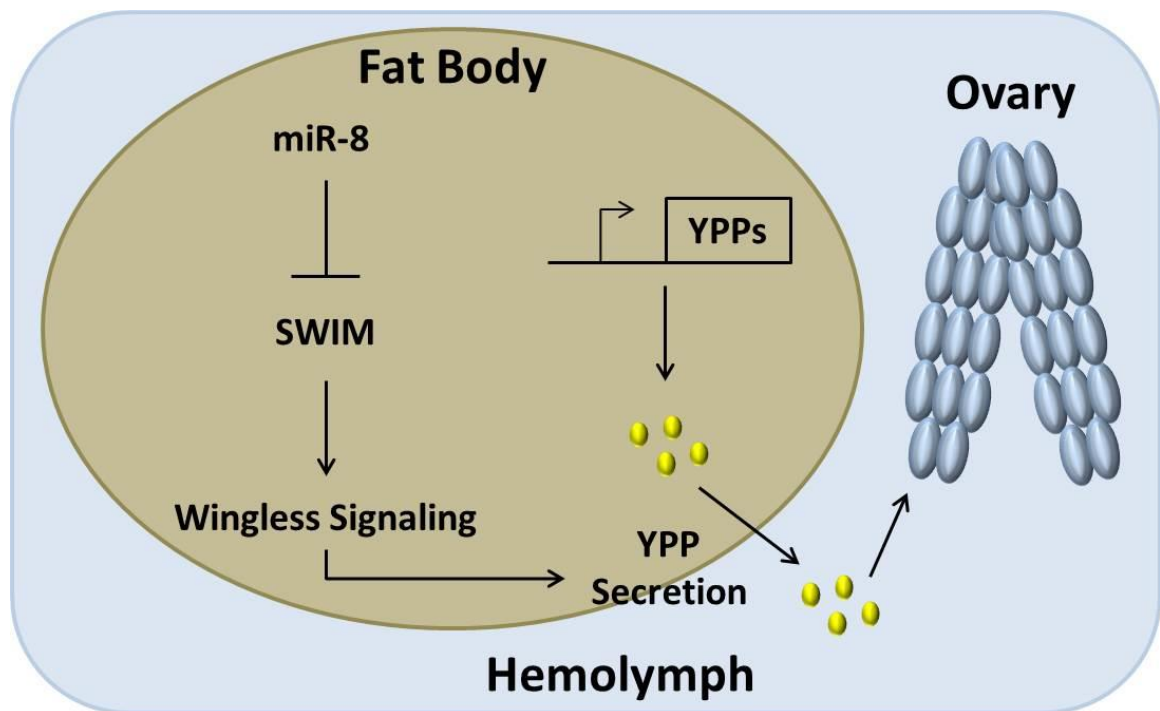


Figure 2.5 Caption

Schematic representation of miR-8 action in the female mosquito fat body post blood meal. miR-8 targets the Wingless signaling pathway through Secreted Wingless-interacting molecule (SWIM) to regulate Yolk Protein Precursor (YPP) secretion by the fat body and uptake into the ovary.

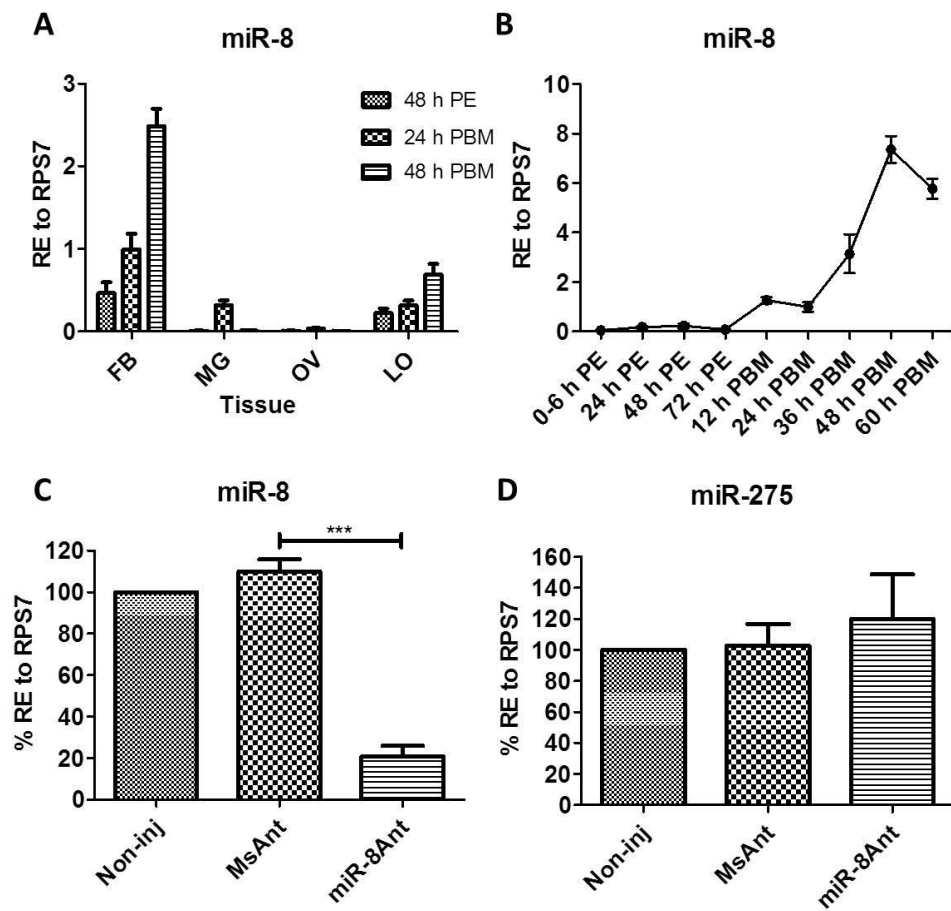
Figure 2.5



Supplemental Figure 2.1 Caption

Mature miR-8 levels are substantially enriched in the female mosquito fat body and successfully depleted by the miR-8 antagomir. (A) Relative expression of mature miR-8 in fat body (FB), midgut (MG), ovary (OV) and left over tissue (LO) at 48 h PE, and 24 and 48 h PBM. (B) Relative expression profile of mature miR-8 in the female mosquito fat body. Relative expression was analyzed at the following time-points: 0-6, 24, 48 and 72 h post eclosion (PE), and 12, 24, 36, 48, 60 h post blood meal (PBM). (C) Percent relative expression of miR-8 in the female mosquito fat body 24 h PBM. (D) Mature miR-275 percent relative expression in the female mosquito fat body 24 h PBM. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

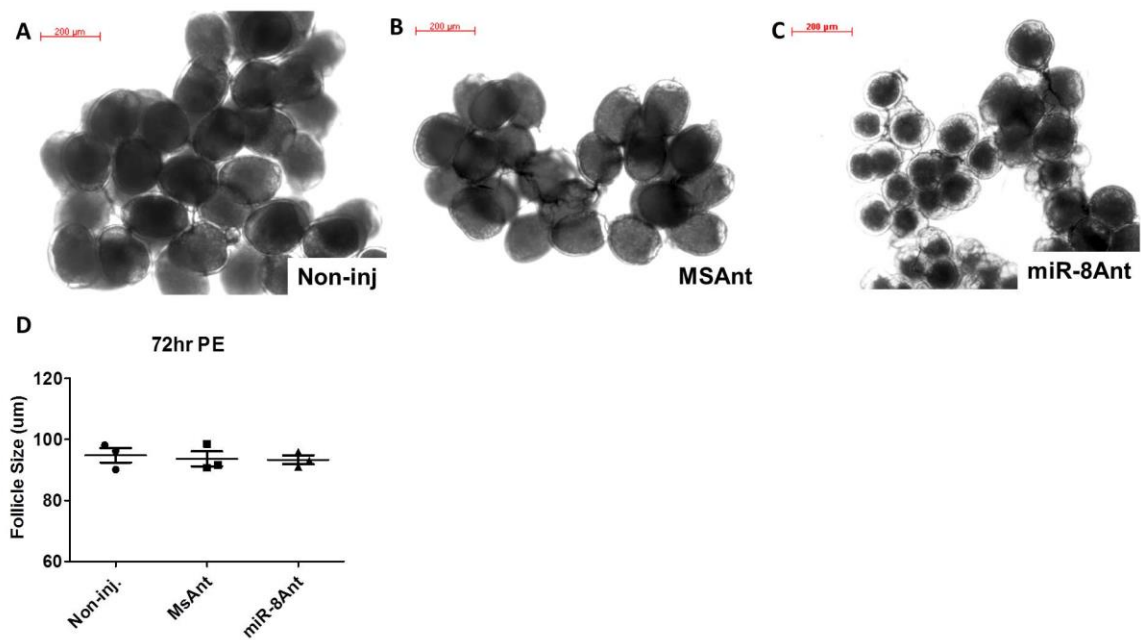
Supplemental Figure 2.1



Supplemental Figure 2.2 Caption

miR-8 antagomir treated female mosquito oocytes. (A-C) Individual ovarian follicles 24 h post blood meal (PBM) of (A) non-injected females. (B) Individual ovarian follicles of MsAnt-treated females. (C) Individual ovarian follicles of miR-8Ant-treated females. Images (A-C) obtained under the same conditions using the 5x objective on the Zeiss Axio Observer.A1 microscope. Scale bar is 200µm. (D) Average follicle size of miR-8Ant-treated, MsAnt-treated and non-injected mosquitoes 72 h post eclosion (PE).

Supplemental Figure 2.2



Supplemental Figure 2.3 Caption

Genomic DNA PCR validation of incorporation and integrity of transgenic

constructs into the *Aedes aegypti* genome, (A) UAS-miR8-SP responder construct and

(B) Vg-Gal4 driver construct modified from Kokoza et al. 2011 (C) Genomic PCR to

confirm responder construct integration for all three independent UAS-miR8-SP (M3-2;

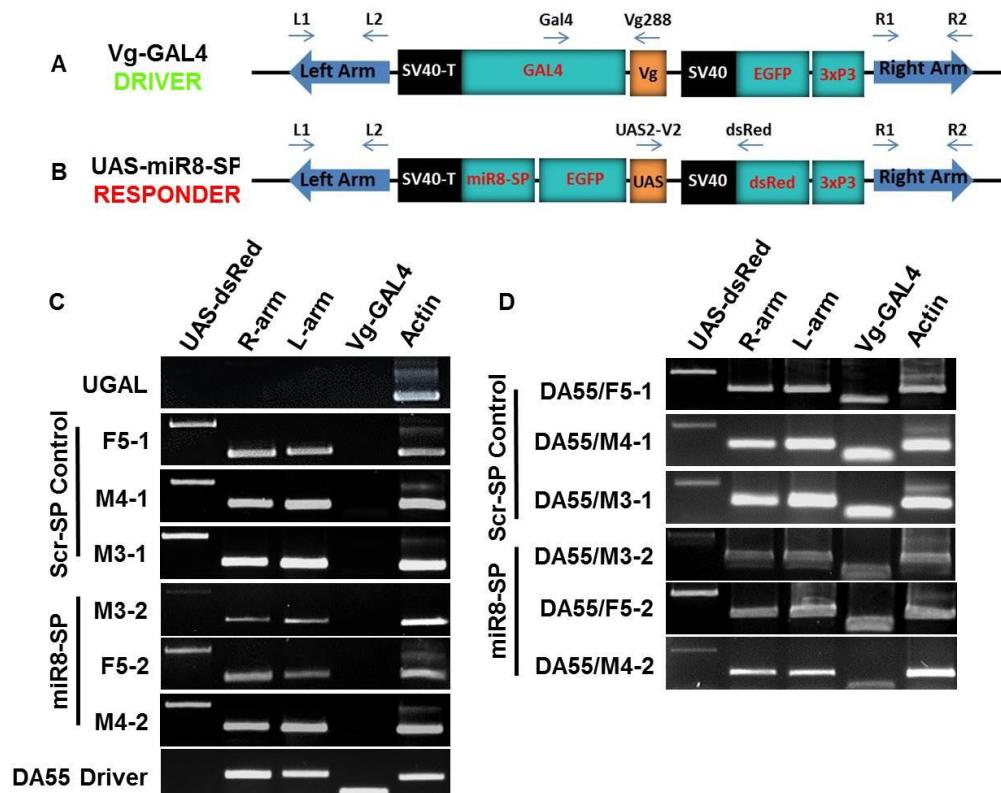
F5-2; M4-2) and UAS-Scr-SP responder lines (F5-1; M4-1; M3-1), the Vg-Gal4 driver

and wildtype (UGAL). (D) Genomic PCR to confirm responder-driver hybrid for all three

Vg-Gal4/UAS-miR8-SP (DA55/M3-2; DA55/F5-2; DA55/M4-2) and Vg-Gal4/UAS-Scr-

SP lines (DA55/F5-1; DA55/M4-1; DA55/M3-1).

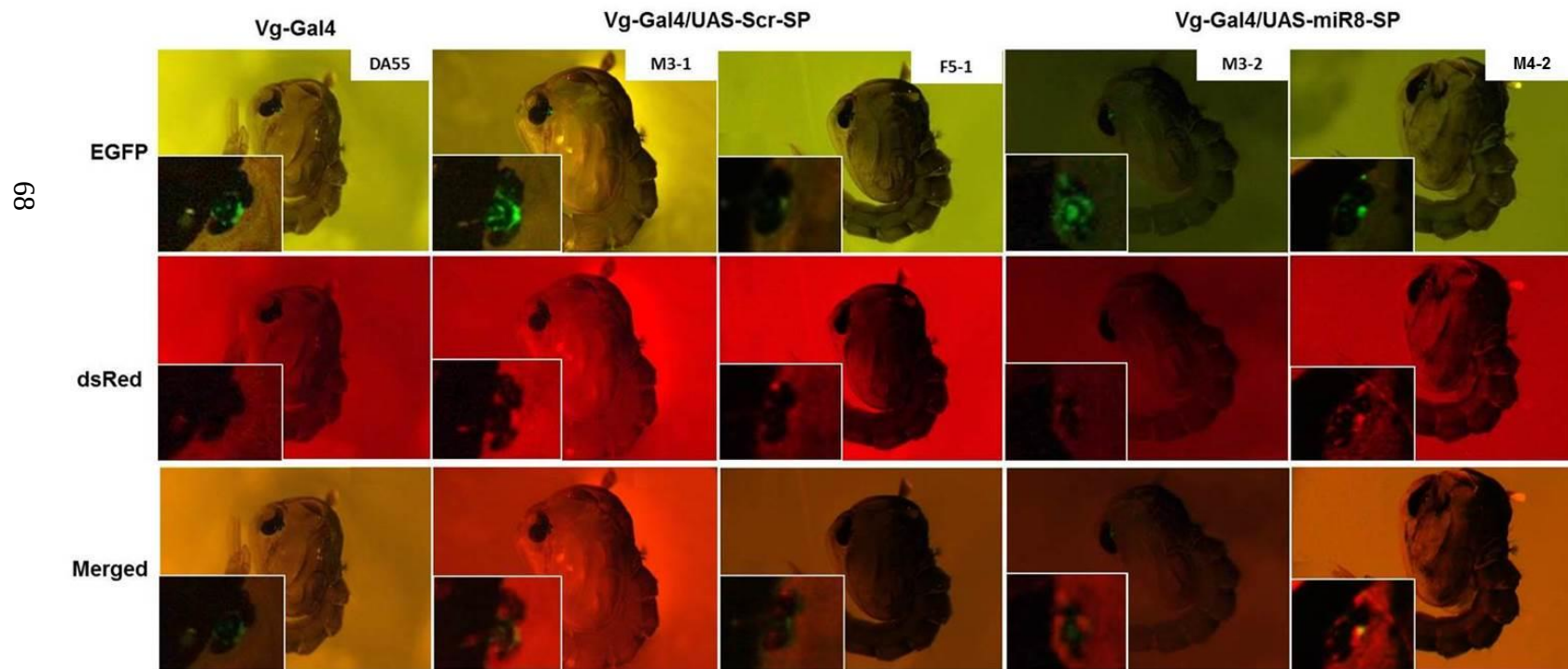
Supplemental Figure 2.3



Supplemental Figure 2.4 Caption

Selection of transgenic Vg-Gal4/UAS-miR8-SP and Vg-Gal4/UAS-miR8-Scr hybrids. Selection was performed on the basis of EGFP and dsRed selectable markers in the eye. Pupa were visualized using the Leica M165FC stereo microscope.

Supplemental Figure 2.4



Supplemental Figure 2.5 Caption

Vg-Gal4/UAS-miR8-SP mosquitoes display delayed ovarian growth, retain eggs and exhibit abnormal melanization. (A-D) Individual ovarian follicles of (A) wild-type

(UGAL), (B) Vg-Gal4 (DA55) driver, (C) Vg-Gal4/UAS-Scr-SP control (DA55/M3-1)

and (D) Vg-Gal4/UAS-miR8-SP (DA55/M4-2). Images (A-D) obtained under the same

conditions using the 5x objective on the Zeiss Axio Observer.A1 microscope. Scale bar is

100µm. (E) When female ovaries were observed at 5 days post blood meal (PBM), Vg-

Gal4/UAS-miR8-SP (DA55/M4-2) continued to retain eggs compared to Vg-Gal4/UAS-

Scr-SP (DA55/M3-1) control and wild-type (UGAL) lines. (F) At 5 days PBM, Vg-

Gal4/UAS-miR8-SP (DA55/M4-2) lines continue to retain eggs to varying degrees, and

ovaries began to undergo melanization - a process that is normally observed after

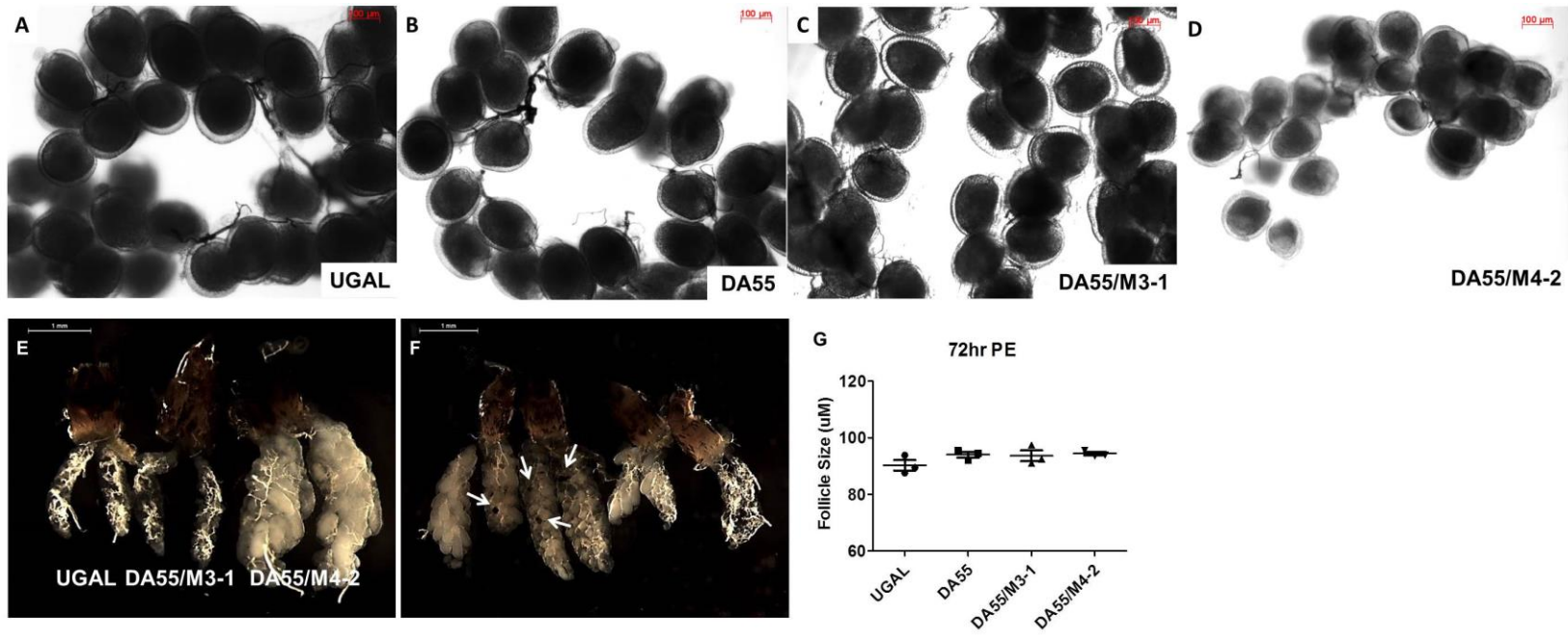
oviposition. (E-F) Ovaries were visualized using the Leica M165FC stereo microscope.

Scale bar is 1mm. (G) Average follicle size of Vg-Gal4/UAS-miR8-SP (DA55/M4-2)

miR-8 depleted females compared to Vg-Gal4/UAS-Scr-SP (DA55/M3-1), Vg-Gal4

(DA55) driver and wild-type controls 72 hr PE.

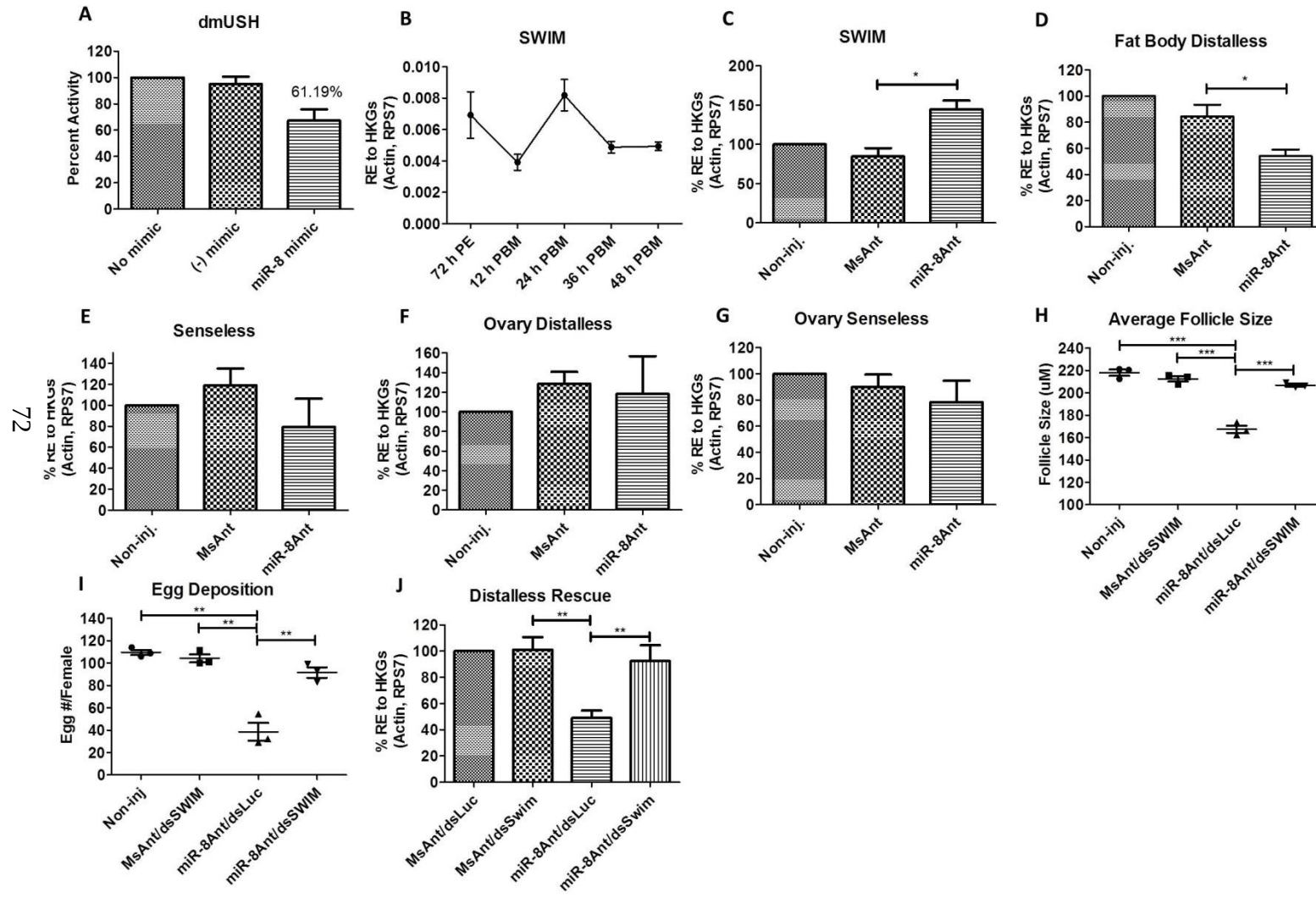
Supplemental Figure 2.5



Supplemental Figure 2.6 Caption

miR-8 directly targets AAEL001232. (A) Relative expression profile of *swim* in the female mosquito fat body. Relative expression was analyzed at the following time-points: 72 h post eclosion (PE), and 12, 24, 36, 48 h post blood meal (PBM). (B) Dual Luciferase Reporter Assay positive control CG2762 *Drosophila* U-shaped (USH), a validated miR-8 target. (C) AAEL001232 relative expression increases in miR-8Ant-treated female mosquitoes. (D) Long range Wingless signaling is reduced in miR-8Ant-treated female mosquito fat body. (E) Short range wingless signaling is unaffected in the miR-8Ant-treated female mosquito fat body. (F-G) Wingless signaling is unaffected in the miR-8Ant-treated female mosquito ovaries. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$. (H) Swim RNAi restores ovary development phenotype of miR-8Ant mosquitoes. (I) Swim RNAi restores egg deposition phenotype of the miR-8Ant mosquitoes. (J) Swim RNAi restores long-range Wingless signaling inhibition in miR-8Ant mosquitoes. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Supplemental Figure 2.6

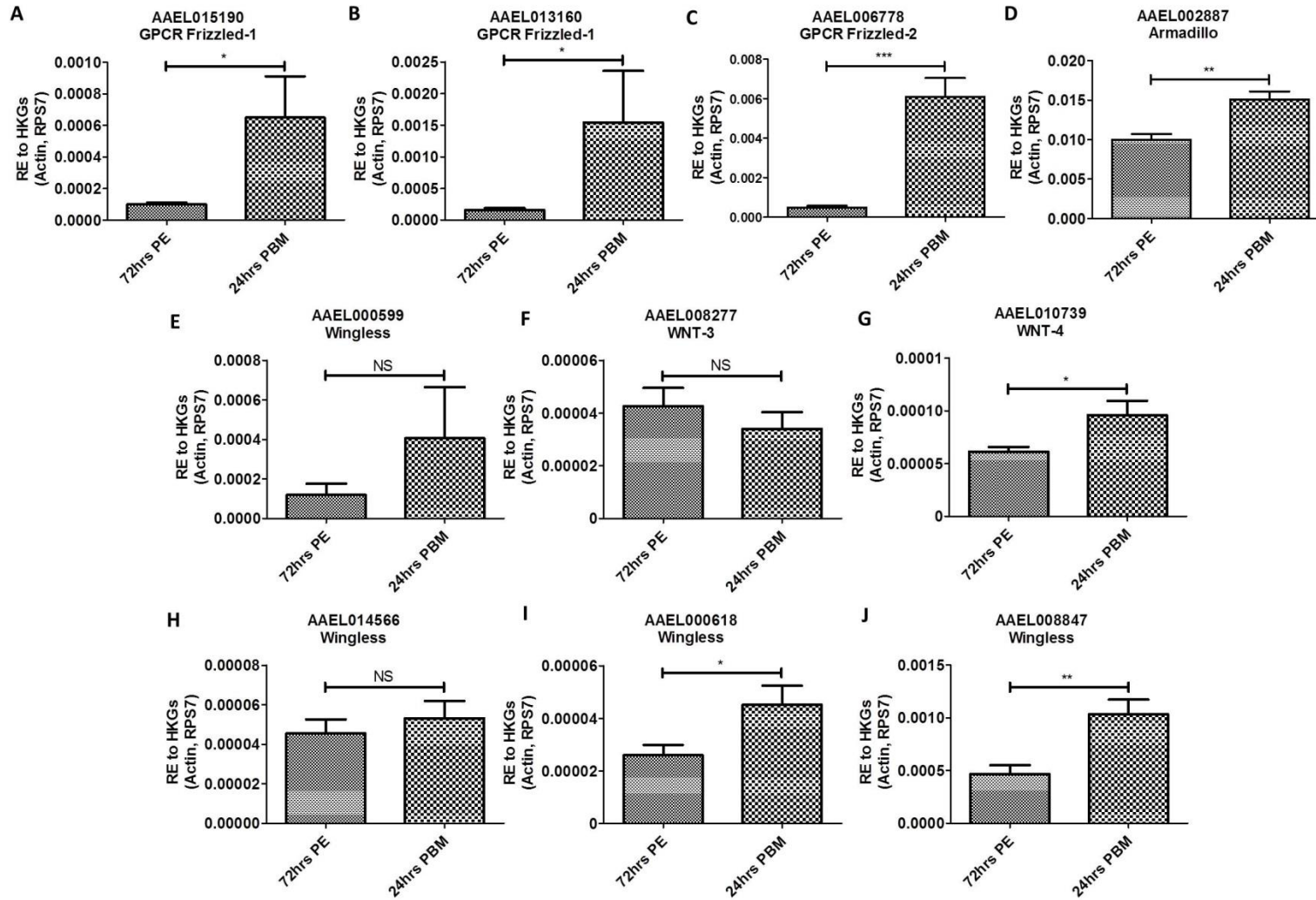


Supplemental Figure 2.7 Caption

The expression of Wingless signaling pathway components increase post blood meal.

(A-D) Relative expression of Wingless (Wg) receptors (A) Frizzled-1 (AAEL015190), (B) Frizzled-1 (AAEL013160), and (C) Frizzled-2 (AAEL006778) and (D) a downstream component of the Wingless signaling pathway, Armadillo (AAEL002887). Wg signaling pathway components are significantly enriched in the female mosquito fat body post blood meal. (E-J) Relative expression of Wg transcripts in the female mosquito fat body. Wg precursors are produced in the female fat body and significantly enriched post blood meal. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Supplemental Figure 2.7



Supplemental Figure 2.8 Caption

miR-8 depleted females display a disruption in yolk protein precursor secretion and lipid accumulation. (A) Vitellogenin gene expression 24 h post blood meal (PBM) (B) Lipophorin gene expression 24 h post blood meal (PBM). Expression calculated against housekeeping genes (HKGs): RPS7 and Actin. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Coomassie blue stain of protein gel with 2 ug of protein from each sample, demonstrating equal loading. Total protein was quantified using the Bio-Rad Protein Assay (D-F) DAPI staining of nuclei in (D) miR-8Ant, (E) MsAnt and (F) non-injected female ovaries. Images D-F obtained under the same conditions. (G-I) Nile Red staining of lipids in (G) miR-8Ant, (H) MsAnt and (I) non-injected female ovaries. Images D-I obtained under the same conditions and visualized on Leica SP5 confocal microscope using the 20x objective lens. Scale bar is 100 μ m.

Supplemental Figure 2.8

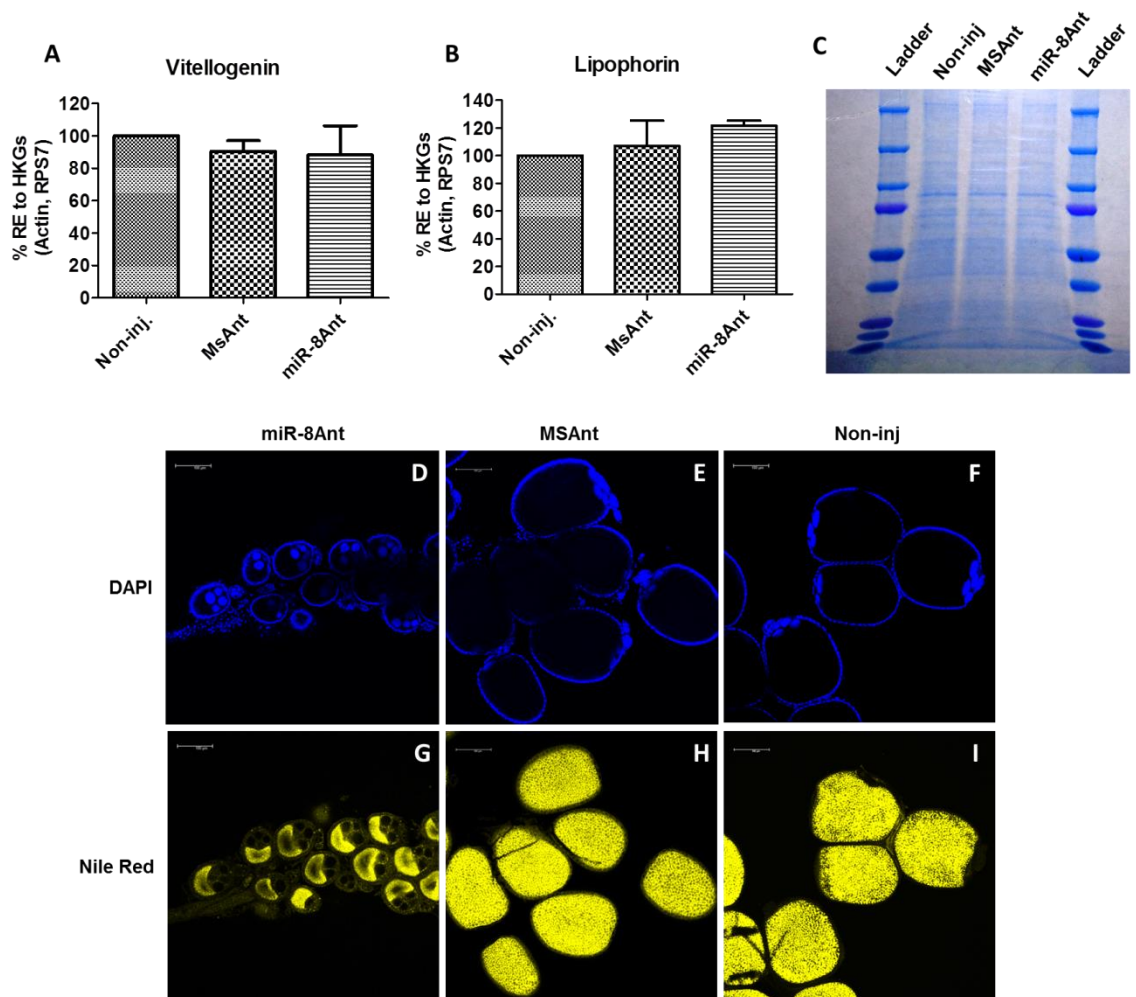


Table 1: Multi-algorithm miR-8 target prediction in mosquito 3'UTRs

Gene ID	Gene name	Species	Algorithm*
AAEL003834-RA	Metalloproteinase, putative	<i>Aedes</i>	IN; TS; PITA; R; RH
AAEL000454-RA; RB	Isocitrate dehydrogenase	<i>Aedes; Anopheles</i>	IN; TS; PITA; R
AAEL001232-RA	Tubulointerstitial nephritis antigen [†]	<i>Aedes; Anopheles</i>	IN; TS; PITA; R
AAEL008347-RA	Monocarboxylate transporter	<i>Aedes; Anopheles</i>	IN; TS; PITA; R
AAEL005958-RA	Oxidoreductase	<i>Aedes</i>	IN; PITA; R; RH
AAEL009059-RA	arp2/3 complex 16 kd subunit (P16-arc)	<i>Aedes</i>	IN; PITA; R; RH
AAEL009530-RA	tmc6 protein (evin)	<i>Aedes</i>	IN; PITA; R; RH

*Algorithms used include in-house (IN), TargetScan (TS), PITA, miRanda (R), and RNAhybrid (RH).

[†]Homolog to *Drosophila* Swim.

CHAPTER III

Mosquito-Specific MicroRNA-1174 Targets *serine hydroxymethyltransferase* to Control Key Functions in the Gut

3.1 Abstract

Lineage-specific microRNAs (miRNAs) may contribute to functions specific to hematophagous mosquitoes and, as such, have potential for contributing to the development of future mosquito control approaches. Here we report that the mosquito- and gut-specific miRNA, miR-1174, is required for proper sugar absorption, fluid excretion, blood intake, and, consequently, egg maturation and survival in female mosquitoes. miR-1174 is highly expressed and localized in the posterior midgut, the blood-digesting portion of the mosquito alimentary canal. Depletion of miR-1174 results in severe defects in sugar absorption and blood intake. We identified *serine hydroxymethyltransferase* (*SHMT*) is a direct miR-1174 target. The adverse phenotypes caused by miR-1174 silencing were rescued by *SHMT* RNA interference. Our results suggest that miR-1174 is essential for fine-tuning the *SHMT* transcript to levels necessary for normal mosquito gut functions.

3.2 Introduction

Females of the vector mosquito species have evolved obligatory blood-feeding requirements to supply amino acids and other nutrients for egg development (1). Consequently, mosquitoes serve as vectors of many devastating human diseases. If a mosquito acquires a blood meal from an infected host, disease pathogens will enter the mosquito gut with ingested blood. The mosquito gut plays an important functional role in blood digestion and as a first line of defense against pathogens. Hence, understanding the molecular basis of gut physiology is of great significance for the development of future approaches toward vector and pathogen control (2-4).

MicroRNAs (miRNAs) are noncoding 22 to 23 nucleotide RNAs that function to repress translation and promote transcript decay (5-7). Most animal miRNAs imperfectly pair with complementary sites within the 3' untranslated regions (UTR) of messenger RNA (8, 9). The mosquito genome gives rise to over 100 conserved and lineage-specific miRNAs. Lineage-specific miRNAs may contribute to developmental novelties in blood-feeding mosquito species and represent attractive targets for vector control approaches. Although several mosquito miRNAs have been characterized functionally (10-14), little is known regarding the role of mosquito-specific miRNAs and their contribution to mosquito-specific events (15).

Many miRNAs are elevated in the female mosquito gut after a blood meal, suggesting that they regulate blood-digestive events (16-18). Previously, the mosquito-specific miR-1174/miR-1175 microRNA (miRNA) cluster was shown to be highly expressed in the *Anopheles gambiae* gut post blood meal (PBM) (16); however, its role

remained undetermined. In this report, we examine the role of the mosquito- and gut-specific miR-1174/miR-1175 cluster and present evidence that miR-1174 and its target, *serine hydroxymethyltransferase* (*SHMT*), control key gut functions in the yellow fever mosquito, *Aedes aegypti*.

3.3 Materials and Methods

Mosquito Rearing

Mosquito larvae were reared at 27 °C in water supplemented with a mixture of yeast and rat chow (1/1 ratio). Adult *Ae. aegypti* mosquitoes were maintained at 27 °C and 80% humidity in cages with unlimited access to 10% sugar solution and water until blood feeding. Three- to four-day-old female *Ae. aegypti* mosquitoes were blood fed on white rats. Laboratory vertebrate animals were kept according to National Institutes of Health (NIH)-approved conditions at a certified facility at the University of California, Riverside. Mosquito tissues were dissected in the *Aedes* physiological solution (19).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). DNA was synthesized using Platinum PCR SuperMix High Fidelity (Invitrogen). Mature miRNA and mRNA cDNA was produced using the miScript II RT Kit (Qiagen). mRNA expression was analyzed quantitatively using the QuantiTect SYBR Green PCR kit

(Qiagen), and mature miRNA expression was measured using the miScript SYBR Green PCR Kit (Qiagen).

Antagomir and dsRNA Treatments

The dsRNA for *serine hydroxymethyltransferase* was synthesized using MEGAScript T7 (Ambion). The dsRNA for luciferase (dsLuc) was used as a negative control. Mosquitoes were injected with 2 µg of dsRNA in 0.3–0.5 µL distilled water at 12 h post eclosion (PE). Transcript abundance was analyzed by means of qRT-PCR analysis, as described above. RNAi-treated mosquitoes were maintained on 10% (wt/vol) sucrose solution for 4 d and then given a blood meal. Transcript abundance was examined in the gut tissue isolated from RNAi-treated mosquitoes at 24 h PBM by means of qRT-PCR. RNAi depletion of luciferase (dsLuc) served as a control.

Antagomirs were purchased from Dharmacon and designed using the RNA module for custom single-stranded RNA synthesis available at www.dharmacon.com/rna/rna.aspx. Antagomirs were designed as previously described (10). Antagomir to miR-1174 was 5' mA(*)mU(*)mGmGmGmUmAmUmUmAmAmGmUmAmGmAmU(*)mC(*)mU(*)mG(*)mA-Chl3'. Mosquitoes were anesthetized with CO₂ 12 h PE and microinjected into the thorax at a dose of 50 pmol per mosquito (200 µM in a volume of 0.25 µL). Mosquitoes were given a 4-d recovery period before phenotype analysis or blood feeding.

Computational Prediction of microRNA Targets

For the prediction of miR-1174 targets in *Ae. aegypti* and *An. gambiae*, we used five different target prediction programs. The features related to the miRNA/mRNA binding site interactions that these algorithms use were taken into account while selecting the programs. The readily available miRNA target prediction programs used for this analysis are PITA (20), miRanda (21), RNAhybrid (22), and TargetScan (23). In addition, we used a program that has been developed in-house. The algorithm for the “in-house” program is a relaxed implementation of the five features proposed by Grimson et al. (24): (i) perfect seed-matching; (ii) additional Watson–Crick base pairing in nucleotides 12–17 (complementary seed site); (iii) location in locally Adenine-Uracil (AU)-rich regions; (iv) sites in the first 15 nucleotides of the 3' UTR are seemingly less effective; and (v) when long 3' UTR's are divided into quartiles, sites that reside within the first and last quartiles of the 3' UTR are more efficient. The program identifies all seed match sites and assigns scores on the basis of the features mentioned above. For example, one point is scored if the starting nucleotide of the seed region of a miRNA is beyond nucleotide 15 in the selected 3' UTR. For each nucleotide within the 3' complementary motif (nucleotides 13–16) of a miRNA that matches the sequence on the targeted transcript, 0.25 points are scored (this includes Guanine-Uracil wobbles). If the seed region target resides inside the first or last quartile of the 3' UTR, another point is scored. Iteratively, 30 nucleotides upstream and downstream from the seed-matching site are scanned on the 3' UTR, and As or Us are registered and scored according to the following formula: $0.25/(\text{distance from seed region edge})$. Thus, if the first nucleotide

upstream or downstream is A or U, the score increases 0.25 points (0.25/1), whereas the score increases by 0.125 points (0.25/2) if the second one is an A or U, and so on, until all 30 nucleotides are analyzed. All points based on the different criteria are summed up, and the sites are ranked according to the scores—the higher the score, the more likely it is that the seed match is a putative miRNA target site. To account for the cross-species conservation, we identified *An. aegypti* genes that have orthologs in *An. gambiae* by reciprocal best BLAST. The 3' UTRs of *An. aegypti* and *An. gambiae* genes were extracted using the information provided in their respective General Feature Format files. Either the mature miRNA sequence or the seed sequence was used, depending on the program requirements.

Cell Culture and Luciferase Assay

Drosophila S2 cells (Invitrogen) were kept at 28 °C in Schneider *Drosophila* medium supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco, Life Technologies) and 1× Antibiotic-Antimycotic (Gibco, Life Technologies). Luciferase constructs were made by inserting the *Ae. aegypti* putative target 3' UTR into the multiple cloning region located downstream of the *Renilla* translational stop codon within the psiCheck-2 vector (Promega). A “miR-1174 synthetic sensor” positive control was also produced by cloning a construct containing three sites reverse complementary to miR-1174 with four nucleotide linkers between each miR-1174 binding site into the multiple cloning region located behind the *Renilla* luciferase stop codon in the psiCheck-2 vector. 100 ng of psiCheck-2 reporters and synthetic *Aedes* miR-1174 miScript miRNA Mimic

(Qiagen) or AllStars Negative Control siRNA (Qiagen) at a final concentration of 100 nM were co-transfected into *Drosophila* S2 cells using Attractene Transfection Reagent (Qiagen). A “no siRNA” treatment control was also used. Dual Luciferase Reporter Assay was performed 48 h post transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase in the psiCheck-2 Vector was used for normalization of *Renilla* luciferase expression. Treatments were made in triplicate, and transfections were repeated three times.

Statistical Analysis

Data were analyzed with GraphPad using Student *t* tests. Probability values of less than 0.05 are considered significant. Data are presented as mean and the SEM.

Phenotypic Rescue Experiments

Double-stranded RNA (dsRNA) was synthesized following a method described previously (19). In brief, dsRNA to SHMT was synthesized using the MEGAscript kit (Ambion). Mosquitoes were anesthetized with CO₂ at 12 h PE, and a mixture containing 2 µg/µL dsRNA and 200 µM antagomir was microinjected into the thorax at a volume of 0.5 µL. Luciferase (Luc) dsRNA was used as a negative control. Mosquitoes were allowed to recover for 3–4 d before blood-feeding.

miRNA in situ Hybridization

Whole-mount in situ hybridizations were performed using a 5' and 3' digoxigenin (DIG)-labeled locked nucleic acid-modified DNA oligonucleotide (LNAs) complementary to miR-1174 (miRCURY LNA Detection probe, Exiqon). The miR-1174 LNA had the following sequence: 5'-ATGGGTATTAAGTAGATCTGA-3'. The Scramble-miR negative control LNA was provided by Exiqon. Female mosquito digestive systems were harvested at 72 h PE and fixed in 4% (vol/vol) paraformaldehyde at room temperature for 1 h with shaking. After 30 min, fixing solution was removed, and samples were thoroughly washed in PBST (1×PBS/0.1%Tween20). Prehybridizations were performed at 55°C for 2h with shaking in prewarmed hybridization buffer [50% (vol/vol) formamide, 5×SSC, 10% (vol/vol) Tween-20, 30µg/mL heparin]. Hybridization was performed at 55 °C with shaking overnight with 25 pmol LNA probe diluted 1:100 in prewarmed hybridization buffer. After hybridization, samples were thoroughly washed in prewarmed hybridization buffer multiple times over a 12-h period and then washed in PBST. Samples were incubated overnight at 4 °C with shaking in Anti-Digoxigenin–AP (Roche) diluted 1:1,500 in PBST. After washing, samples were incubated in alkaline phosphatase staining buffer (100 mM Tris, 150 mM NaCl, 1 mM MgCl₂, pH 9.5) and developed using 1-Step nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt solution (Pierce) according to the manufacturer's protocol.

3.4 Results

Expression analysis of the miR-1174/miR-1175 cluster in the female mosquito midgut

An ortholog of aga-miR-1174 is present in *Ae. aegypti* and differs from that in *An. gambiae* by 2 nucleotides. Similarly, genomic clustering of miR-1174 and miR-1175 is conserved—located at the same locus 83 nucleotides apart (Fig. 3.1A)—suggesting that these two miRNAs may be coregulated. We measured mature miR-1174 and miR-1175 relative expression levels in the midgut of the female mosquito by quantitative real-time (qRT) PCR analysis. *Ae. aegypti* miR-1174 and miR-1175 exhibit similar patterns of expression, reaching their peak levels at 36 h PBM and decreasing to background levels by 72 h PBM (Fig. S3.1A), further supporting co-regulation. Expression of both miRNAs was gut-specific in *Ae. aegypti* (Fig. 3.1B, S3.1B). Moreover, *in situ* hybridization indicated that miR-1174 was localized in the posterior midgut, the part of the alimentary canal where the ingested blood is digested (Fig. 3.1C, S3.1D). High levels of expression of miR-1174 and miR-1175 in the female mosquito midgut PBM suggest that these miRNAs may play an important role in regulating midgut functions related to blood digestion.

miR-1174 depletion results in severe defects in sugar absorption

To determine the function of miR-1174 in mosquitoes, we inhibited miR-1174 by antisense oligonucleotides (antagomirs, Ant-1174) (25). We injected *Aedes* female mosquitoes with 100 pmol of the Ant-1174 at 12 h PE. The level of miR-1174 significantly decreased after its specific inhibition, whereas no change was observed after

the injection of a scrambled oligonucleotide sequence antagomir (Ant-Ct) (Fig. 3.1D). There was no change in the miR-1175 level in Ant-1174-treated mosquitoes (Fig. S3.1C). When maintained on a 10% (wt/vol) sugar solution for 3 d post injection (PIJ), over 20% of the Ant-1174-treated mosquitoes had bloated abdomens (bloated-abdomen phenotype, BAP) (Fig. 3.2A). The BAP was mostly due to the extended fluid-filled crop, a part of the mosquito digestive system used for storing nectar under normal conditions (Fig. 3.2B). Ant-Ct-treated female mosquitoes maintained under the same conditions had a normal appearance, similar to that of WT and H₂O-treated mosquitoes, and their crops were not extended (Fig. 3.2C-G). The weight of each Ant-1174-treated female mosquito increased from ~2.5 mg before injection to 3.3 mg after being maintained on a 10% sugar solution for 3 d PIJ, whereas the weight of Ant-Ct-treated mosquitoes and WT did not change (Fig. 3.2H). Female mosquitoes with the BAP were unable to fly or feed on blood. However, when Ant-1174-treated mosquitoes were maintained only on water for 3 d PIJ, they had normal appearance and behavior similar to those of WT untreated, H₂O-treated, and Ant-Ct-treated mosquitoes. Inhibition of miR-1174 affected the life span of female mosquitoes with 80% mortality within 30 d compared with 30% in Ant-Ct-treated mosquitoes (Fig. 3.2I). We injected 40 pmol of antagomir into male *Aedes* mosquitoes due to their smaller size and found that over 20% Ant-1174-treated males displayed BAP.

miR-1174 depletion results in severe defects in blood digestion

Next, we tested whether Ant-1174-treated female *Ae. aegypti* mosquitoes that did not exhibit the BAP could feed on blood. Only about 20% of these mosquitoes took blood, compared with over 80–90% of Ant-Ct-treated or WT-untreated females (Fig. 3.3A), and the weight per Ant-1174-treated mosquito was significantly lower than that of WT or Ant-Ct-treated mosquitoes at 0.5 h PBM (Fig. 3.3B). Examination of WT and Ant-Ct-treated female mosquitoes at 1.0 h PBM showed that the ingested blood bypassed the crop and was directed into the posterior midgut (Fig. 3.3C-D). In contrast, blood was mostly present in the crop of the Ant-1174-treated female mosquitoes, indicating that blood was directed to the crop of these mosquitoes during feeding (Fig. 3.3E). Unlike WT and Ant-Ct-treated female mosquitoes at 24 h PBM (Fig. 3.3G-H), most of the blood was retained in the crop of Ant-1174-treated mosquitoes (Fig. 3.3I). Inhibition of miR-1174 in female mosquitoes arrested egg development (Fig. 3.3F). These mosquitoes laid significantly fewer eggs than the WT and Ant-Ct-treated mosquitoes (Fig. 3.3J). These results provide evidence that miR-1174 is essential for proper blood intake and, consequentially, egg development in the female mosquito.

Computational and in vitro analysis indicates miR-1174 targets serine hydroxymethyltransferase

To identify the putative gene targets of miR-1174, we used five miRNA target prediction tools—Probability of Interaction by Target Accessibility (PITA) (20),

miRanda (21), RNAhybrid (22), TargetScan (23), and an in-house program developed on the basis of the features proposed by Grimson et al. (24). To weigh for putative binding site conservation, we predicted miR-1174 targets using both *Ae. aegypti* and *An. gambiae* 3' UTRs. All programs were run locally, and the targets predicted by each of these programs were mapped against each other to determine the overlaps. A list of top candidate targets was produced on the basis of predictions by multiple algorithms and their conservation (Table 3.1). Targets that were either identified by all five programs in *Ae. aegypti* (AAEL002510 and AAEL012079) or in *An. gambiae* (AAEL001779 ortholog of AGAP005775), or by four different programs in both *Ae. aegypti* (AAEL005411 and AAEL010558) and their *An. gambiae* orthologs (AGAP003892 and AGAP000964), were shortlisted for further analysis. Following computational prediction, we assessed the 3' UTRs of the top five potential targets for their responses to miR-1174 using the Dual Luciferase Reporter Assay. The 3' UTR for each putative miR-1174 target was cloned downstream of the *Renilla* translational stop codon within the psiCheck-2 vector to produce 3' UTR-fused luciferase reporters. When transfected into *Drosophila* Schneider 2 (S2) cells along with miR-1174 mimic, the luciferase reporter containing the full-length 3' UTR of *SHMT* yielded less than 50% luciferase activity compared with the negative control mimic and “no mimic” control samples (Fig. 3.4A-B). Four other tested genes elicited no response (Fig. S3.2B-E). Hence, of the top five candidate targets, only *SHMT* was validated as the miR-1174 target *in vitro*. Luciferase reporter assay was optimized using a synthetic miR-1174 sensor

containing three repeat miR-1174 binding sites cloned downstream of the *Renilla* translational stop codon (Fig. S3.2A).

miR-1174 regulates expression of serine hydroxymethyltransferase in vivo

To determine the effects of miR-1174 on *SHMT* expression *in vivo*, we first assayed for the levels of the *SHMT* transcript and miR-1174 expression in different tissues. The *SHMT* transcript level in the gut was low relative to other tissues at every sampled time point (Fig. S3.3A-C). This is in contrast to the elevated level of miR-1174 in the gut at the same time points of the female mosquito life cycle (Fig. S3.3B-D). However, the *SHMT* transcript level significantly increased in this tissue of Ant-1174–injected mosquitoes (Fig. 3.4C), suggesting that miR-1174 controls the *SHMT* transcript level *in vivo*. To verify this hypothesis, we injected 100 pmol of miR-1174 mimic and observed a significant increase in the level of miR-1174 in the guts of these mosquitoes (Fig. S3.3E), whereas the level of miR-1175 did not change (Fig. S3.3F). Indeed, injection of the miR-1174 mimic caused a significant decrease in gut *SHMT* transcript level (Fig. 3.4D), further validating *SHMT* as an authentic target of miR-1174 *in vivo*.

To further evaluate *SHMT* as the authentic miR-1174 target gene, we conducted phenotypic rescue experiments through the *SHMT* RNAi depletion in Ant-1174–treated mosquitoes. The RNAi of a physiologically relevant gene was expected to alleviate the adverse phenotypes caused by miR-1174 silencing. This approach has been successfully used in rescuing miRNA mutant phenotypes in *Drosophila* (26, 27). RNAi silencing of *SHMT* resulted in severe phenotypes manifested in the inability of female mosquitoes

to normally digest blood, develop, and lay eggs. We then co-injected 100 pmol of Ant-1174 and 100 ng of dsSHMT into female *Ae. aegypti* mosquitoes at 12 h PE and maintained them for 3 d PIJ on a 10% sugar solution. The gut *SHMT* transcript level in these Ant-1174/dsSHMT mosquitoes was as low as that in untreated WT mosquitoes (Fig. 3.4E). The BAP was not observed in sugar-fed Ant-1174/dsSHMT mosquitoes. There was no weight gain in these mosquitoes, unlike those treated with Ant-1174 only (Fig. 3.5A); they exhibited normal behavior and were able to fly. *SHMT* RNAi also rescued the adverse post blood-feeding phenotypes caused by miR-1174 silencing. The Ant-1174/dsSHMT mosquitoes fed normally on blood, and their weight increased significantly post blood-feeding (Fig. 3.5B). No blood was observed in the crop of these mosquitoes, and they laid significantly more eggs than Ant-1174-treated mosquitoes (Fig. 3.5C). Thus, our data demonstrate that *SHMT* is the authentic miR-1174 target contributing to the adverse phenotypes observed in Ant-1174-treated female mosquitoes.

3.5 Discussion

Our results show that depletion of the mosquito- and gut-specific miRNA, miR-1174, results in severe defects linked to proper sugar absorption, fluid excretion, blood intake, and, consequently, egg maturation and survival in *Ae. aegypti*. Using antagomir-based miRNA inhibition in *Ae. aegypti*, this study represents a significant step toward defining regulatory roles of mosquito-specific miRNAs. Our work implicates that miR-1174 functions as a regulator of sugar absorption and blood intake in female mosquitoes by regulating the expression of *SHMT*. Depletion of miR-1174 in *Ae. aegypti* female

mosquitoes resulted in a dramatic bloated-abdomen phenotype, due to an extended fluid-filled crop, when maintained on a 10% sugar solution for 3 d post injection. Females exhibiting this bloated-abdomen phenotype were unable to fly or feed on blood. Additionally, blood-fed miR-1174-depleted female mosquitoes displayed visually striking PBM phenotypes: blood was directed to the crop instead of the gut, and, as a result, primary follicle growth was drastically inhibited.

Additional work by co-author, Dr. Shiping Liu, characterized miR-1174 function in *An. gambiae* female mosquitoes. Similar to miR-1174 depletion in *Ae. aegypti*, *An. gambiae* female mosquitoes treated with an antagomir specific to *An. gambiae* miR-1174 displayed defects in sugar absorption, fluid excretion, blood digestion and egg development. Twenty-five percent of *An. gambiae* female mosquitoes developed the BAP after maintenance on a 10% sugar solution for 3 d PIJ. A similar phenotype in blood intake and digestion was also observed in Ant-1174-treated female *An. gambiae* mosquitoes. Additionally, inhibition of miR-1174 in *An. gambiae* female mosquitoes arrested egg development and these mosquitoes laid significantly fewer eggs compared to controls. Together these results indicate miR-1174 plays a conserved role in regulation of sugar absorption, fluid excretion and blood digestion in both *Aedes* and *Anopheles* mosquitoes. Furthermore, *Ae. aegypti* female mosquitoes treated with an antagomir specific to miR-1175 did not produce any adverse phenotypes, serving as an additional confirmation of the specificity of miR-1174 action.

Using multiple miRNA target prediction algorithms, we identified *SHMT* as the physiological relevant miR-1174 target contributing to the miR-1174 depletion

phenotypes. Dual Luciferase Reporter Assay using a luciferase reporter vector containing *Ae. aegypti* *SHMT* 3' UTR co-transfected along with miR-1174 mimic resulted in a decrease in *Renilla* luciferase activity *in vitro*, whereas *SHMT* RNAi rescue experiments recovered the miR-1174 depletion phenotype *in vivo*. *SHMT* catalyzes the interconversion of serine and glycine, with tetrahydrofolate (THF) serving as the one-carbon carrier in the de novo synthesis of deoxythymidine monophosphate (dTMP) (28, 29). dTMP serves as a precursor of deoxythymidine triphosphate (dTTP), the optimal dTTP cellular level of which is essential for normal replication of nuclear and mitochondrial DNA (30). Folate metabolism is regulated by iron availability, and, in humans, iron deficiency leads to many abnormalities, including intestinal malfunction. The iron-storage protein ferritin enhances *SHMT* mRNA translation rate and, thereby, de novo thymidine biosynthesis (31). Mosquitoes face the opposite task of removing massive amounts of iron from a blood meal to avoid its toxic effect. This is accomplished by the metabolism of iron and the increased expression of iron storage proteins, including ferritin. Maintaining a low *SHMT* level in the mosquito gut is likely of critical importance to counterbalance the iron-stimulating effect. This may be achieved by the direct action of miR-1174 on the *SHMT* transcript, maintaining it at the level required for normal gut functions.

These experiments provide evidence that the lineage-specific miRNA, miR-1174, functions in anautogenous mosquito species to control gut functions essential for sugar absorption, blood intake, and digestion, and, as a consequence, ovary development in two important vectors of human disease. miR-1174 is present in the blood-feeding mosquito

species, *An. gambiae*, *An. stephensi*, and *Ae. aegypti* and not found in the non-blood-feeding mosquito, *Toxorhynchites amboinensis* (18), further supporting that miR-1174 functions in a conserved mechanism essential for blood-feeding in anautogenous mosquito species. Moreover, miR-1175 has been identified in all four mosquito species, *An. gambiae*, *An. stephensi*, *Ae. aegypti*, and *T. amboinensis* (18). Due to the close proximity of miR-1174 and miR-1175 (Fig. 3.1A), it is possible that miR-1174 arose through a duplication event in the miR-1174/miR-1175 cluster. It remains unknown if miR-1174 was lost, evolved beyond recognition, or if a duplication event never occurred in *T. amboinensis*. However, our findings suggest that miR-1174's essential role in blood-feeding and other gut functions placed a strict evolutionary pressure in blood-feeding mosquito species leading to miR-1174 conservation.

3.6 Acknowledgements

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Figure 3.1 Caption

Characterization of miR-1174 and miR-1175 in *A. aegypti*. (A) Genomic organization of miR-1174/miR-1175. (B) Tissue-specific expression of miR-1174. mg, midgut; FB, fat body; OV, ovary; LO, leftover. (C) In situ hybridizations using a 5' and 3' digoxigenin (DIG)-labeled locked nucleic acid-modified DNA oligonucleotide complementary to miR-1174 (1174LNA). (D) miR-1174 level in the whole body after antagomir silencing (Ant-1174) compared with the effect of scrambled control antagomir (Ant-Ct) in female *A. aegypti* mosquitoes. WT shows miR-1174 level in untreated mosquitoes. qRT-PCR, error bars depict $\pm$ SEM.

Figure 3.1

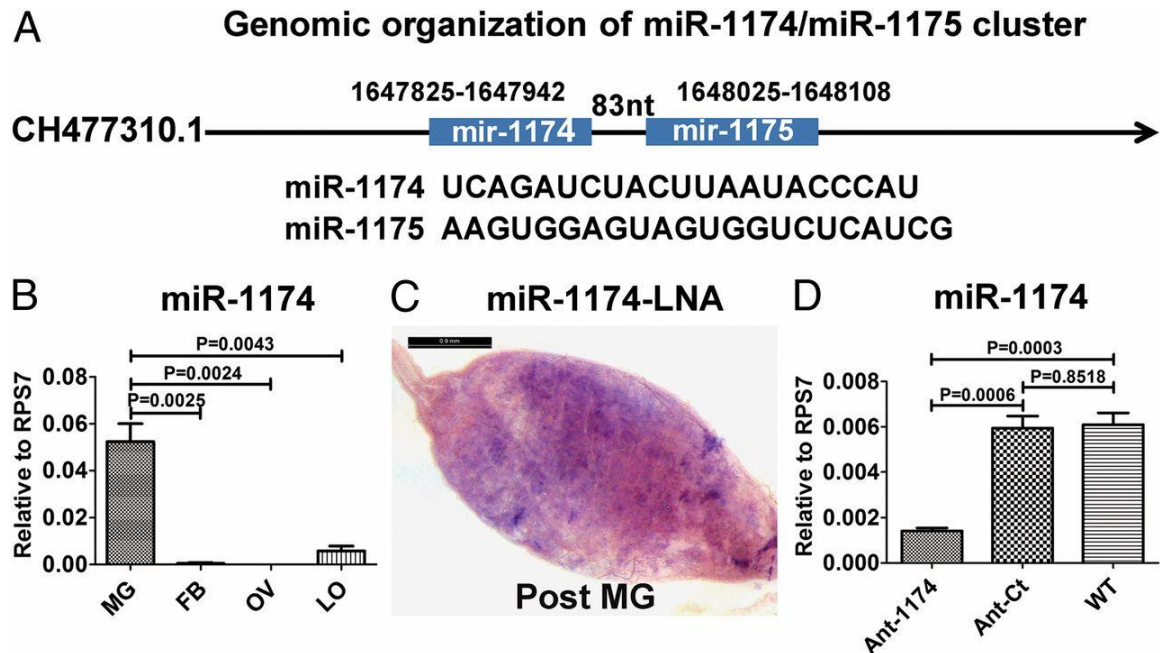


Figure 3.2 Caption

Effect of miR-1174 silencing in *A. aegypti* female mosquitoes maintained on 10% sugar solution. (A) A female mosquito with the bloated-abdomen phenotype after miR-1174 antagomir (Ant-1174) injection. (B) Digestive system isolated from a female mosquito with bloated-abdomen phenotype. Note an extremely extended crop, mg, midgut. (C) A female mosquito injected with control antagomir (Ant-Ct). (D) Digestive system from isolated Ant-Ct-treated mosquito. (E) Untreated WT mosquito. (F) Digestive system isolated from a WT mosquito. (G) Percentage of female mosquitoes injected with Ant-1174, Ant-Ct, H₂O, or untreated WT displaying the bloated-abdomen phenotype. (H) Weight changes per mosquito 3 d PIJ. (I) Survival of female mosquitoes injected with Ant-1174, Ant-Ct, or untreated WT. Error bars depict $\pm$ SEM.

Figure 3.2

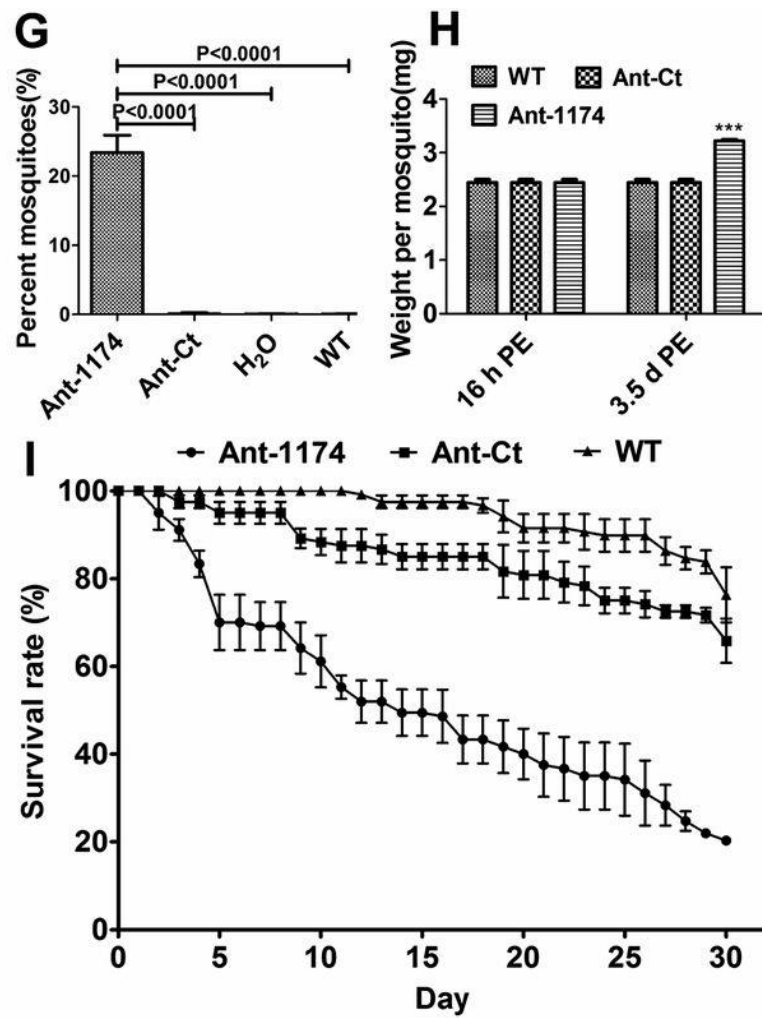
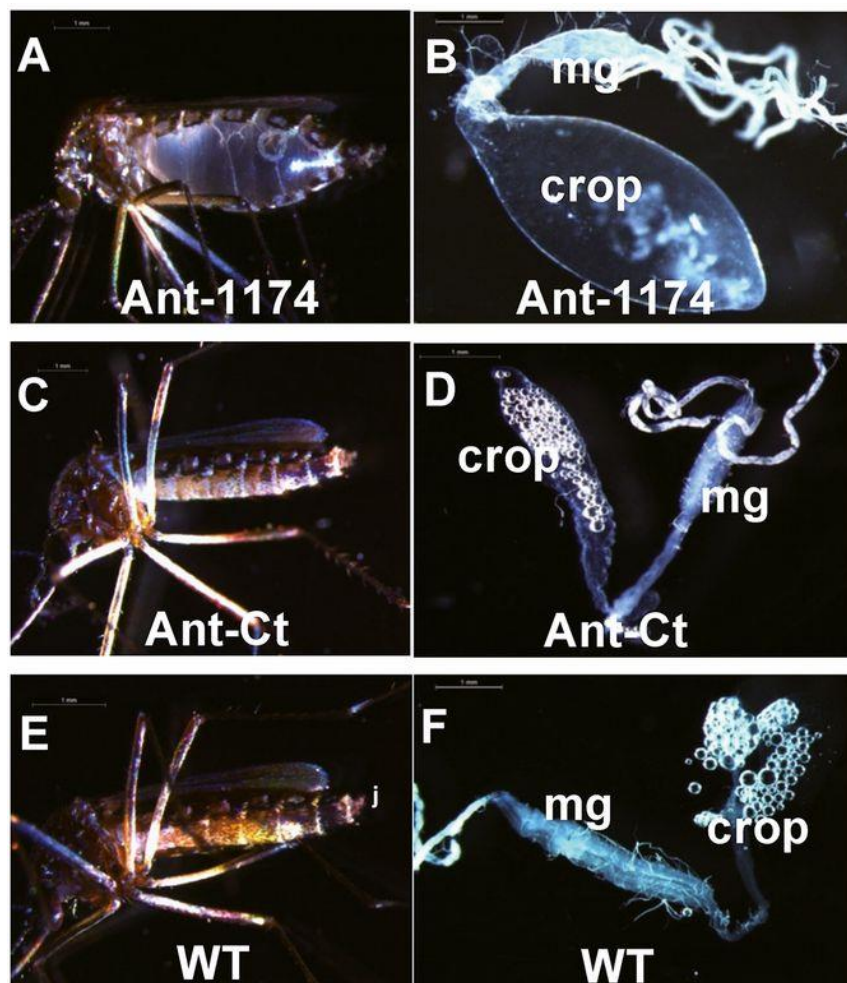


Figure 3.3 Caption

Effect of miR-1174 silencing in blood-fed *A. aegypti* female mosquitoes. (A and B) Mosquitoes that blood-fed post miR-1174 silencing. (A) Percent of mosquitoes for sucking blood. (B) Body weight post blood-feeding. (C–E) Isolated digestive systems from mosquitoes 1 h after blood-feeding. (C) Untreated WT. (D) Ant-Ct–injected. (E) Ant-1174–injected. mg, midgut. (F) Ovaries isolated from female mosquitoes injected with Ant-Ct, Ant-1174, or untreated WT. (G–I) Isolated digestive systems from mosquitoes 24 h after blood-feeding. (G) Untreated WT. (H) Ant-Ct–injected. (I) Ant-1174–injected. (J) Number of eggs per female mosquito injected with Ant-1174, Ant-Ct, or untreated WT. Error bars depict $\pm$ SEM.

Figure 3.3

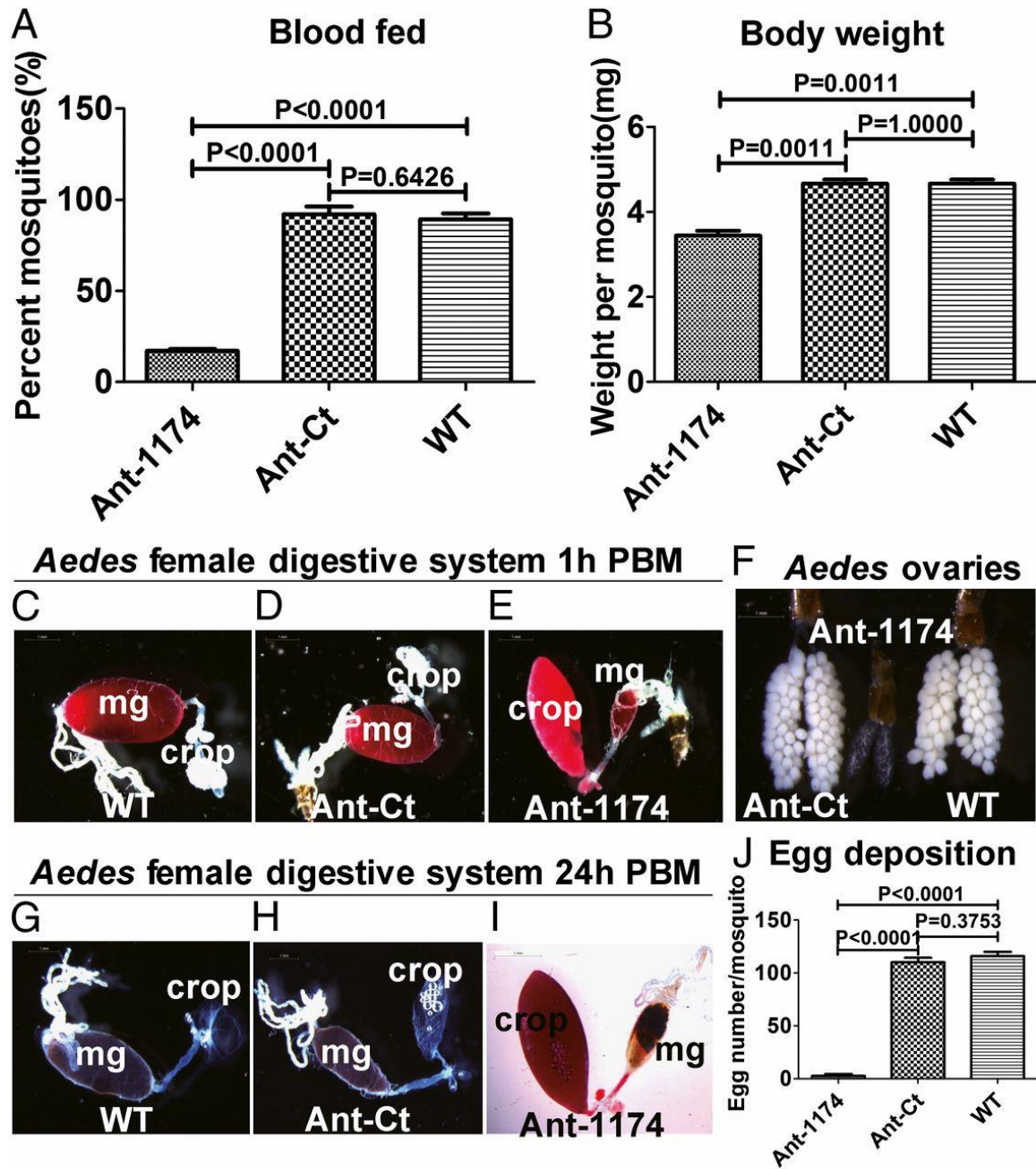


Figure 3.4 Caption

SHMT is a direct target of miR-1174. (A) miR-1174 interaction with the AAEL002510 (SHMT) 3'UTR (B) miR-1174 directly targets SHMT 3'UTR in *in vitro* Dual Luciferase Reporter Assay. Data represent the average percentage activity (Δ Fold Activity $\times$ 100) of triplicate samples. (C) miR-1174 silencing (Ant-1174) results in an increase of the *SHMT* transcript level. Injection of Ant-Ct has no effect on *SHMT* levels. (D) Injection of miR-1174 mimic (Mimic-1174) results in a decrease of the *SHMT* transcript level, similar to that of SHMT RNAi (dsSHMT). (E) Co-injection of Ant-1174 and dsSHMT results in a lower level of the *SHMT* transcript (Ant-1174/dsSHMT). Co-injection of Ant-1174 and control dsRNA (dsLuc) does not affect an elevated level of the *SHMT* transcript caused by miR-1174 silencing (Ant-1174). (C–E) qRT-PCR. Error bars in A–D depict $\pm$ SEM.

Figure 3.4

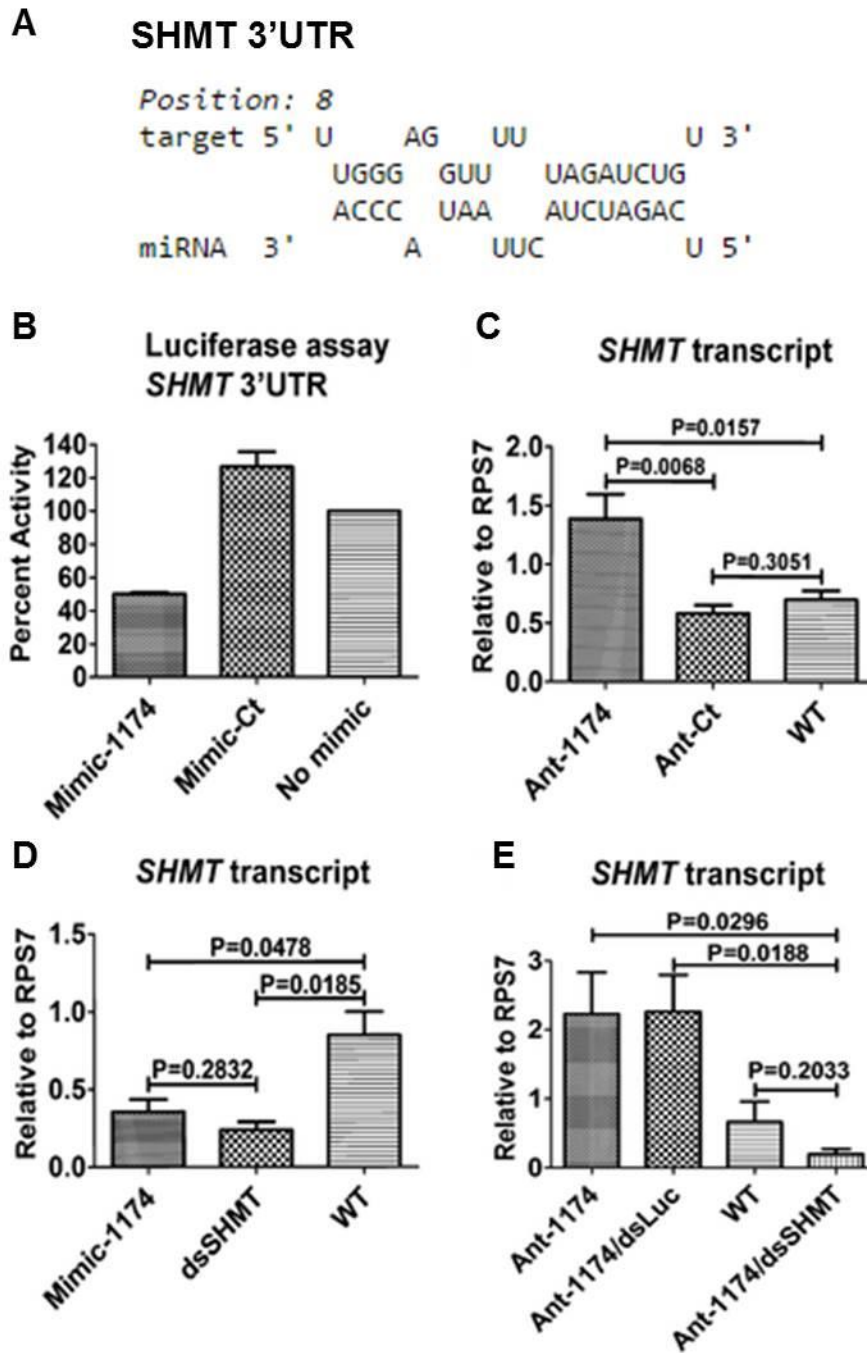


Figure 3.5 Caption

Rescue of phenotypes caused by miR-1174 silencing in female *Aedes* mosquitoes. (A)

Mosquitoes were treated 12 h post eclosion, maintained on 10% sugar solution for 3 d,

and then examined. Increase in weight over that of WT was plotted. (B) Mosquitoes

treated as in A were blood-fed, and their weight was measured. Increase in weight was

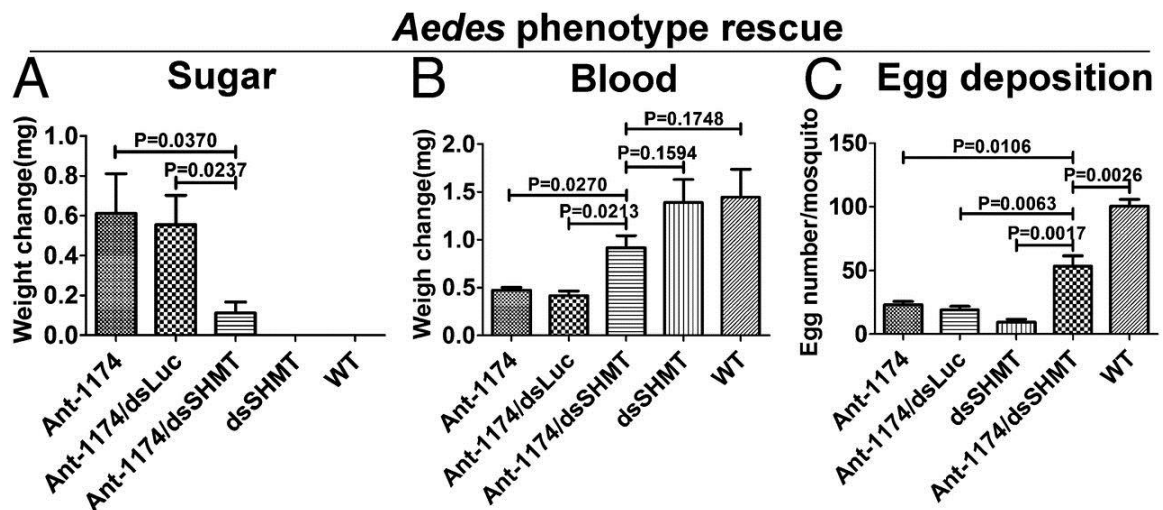
plotted. (C) Number of eggs per female mosquito from experimental treatments in B.

Ant-1174, miR-1174 Antagomir; Ant-1174/dsLuc, co-injection of Ant-1174 with control

Luciferase dsRNA; Ant-1174/dsSHMT, co-injection of Ant-1174 and dsSHMT;

dsSHMT, injection of dsSHMT; and WT is untreated. Error bars in A–C depict $\pm$ SEM.

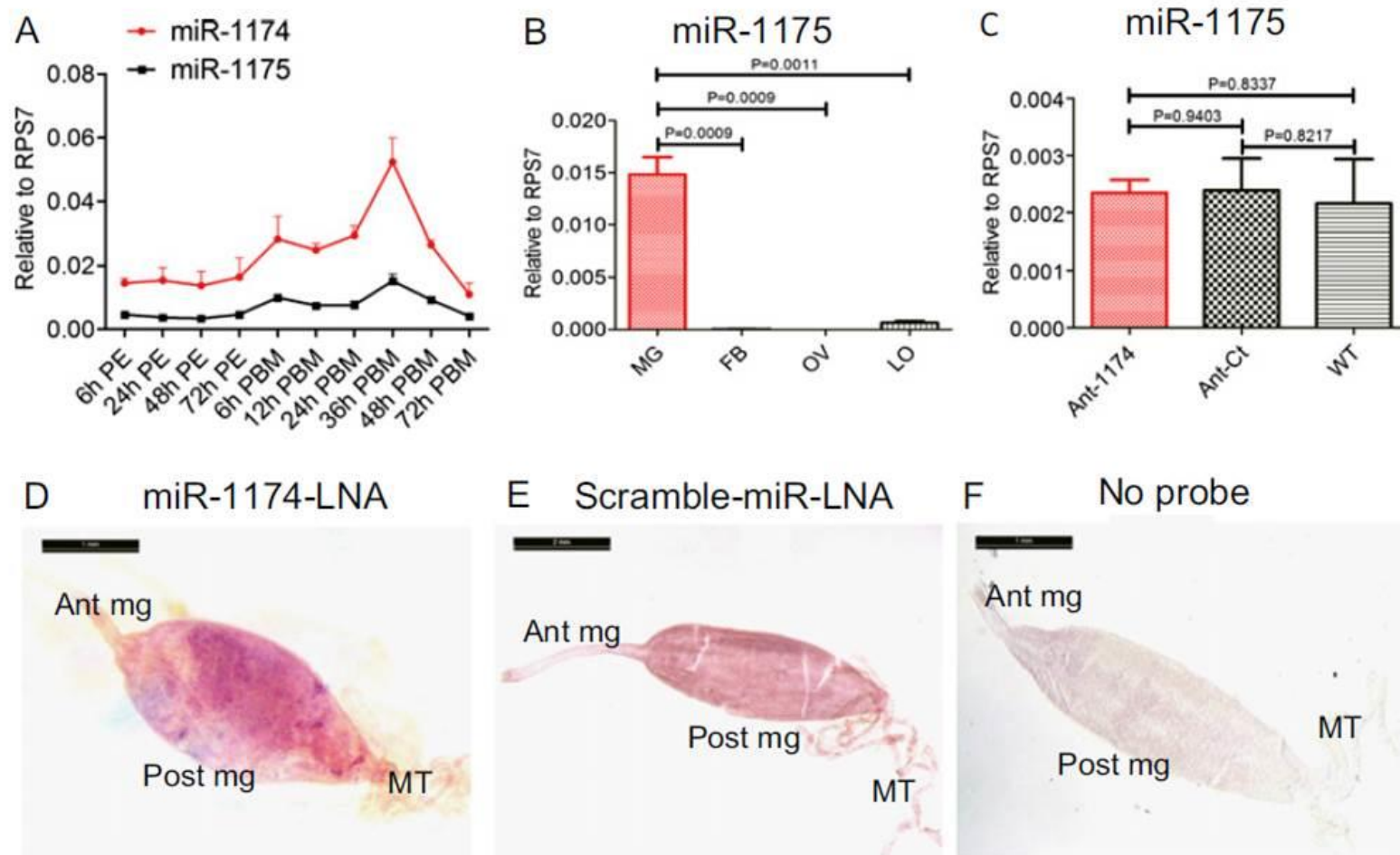
Figure 3.5



Supplemental Figure 3.1 Caption

Expression profiles of miR-1174 and miR-1175 in *Aedes aegypti*. (A–C) Quantitative real-time (qRT) PCR. (A) Expression profiles of miR-1174 and miR-1175 in the midgut during reproductive cycles of female *Ae. aegypti* mosquitoes. PE, h posteclosion; PBM, h post blood meal. (B) Tissue-specific expression of miR-1175. (C) miR-1175 levels in treated, as in B. Error bars depict $\pm$ SEM (D–F) Whole-mount *in situ* localization of miR-1174 in the digestive system of the female *Aedes* mosquito. (D) In situ hybridizations using a 5' and 3' digoxigenin (DIG)-labeled locked nucleic acid-modified DNA oligonucleotide (LNA) complementary to miR-1174 (miR-1174–LNA). (E) The Scramble-miR negative control LNA (Scramble-miR–LNA). (F) Control untreated mosquito. The digestive system of female mosquitoes consists of anterior midgut (Ant mg), posterior midgut, and stomach (Post mg). The excretory system attached to the digestive system is represented by the Malpighian tubules (MT).

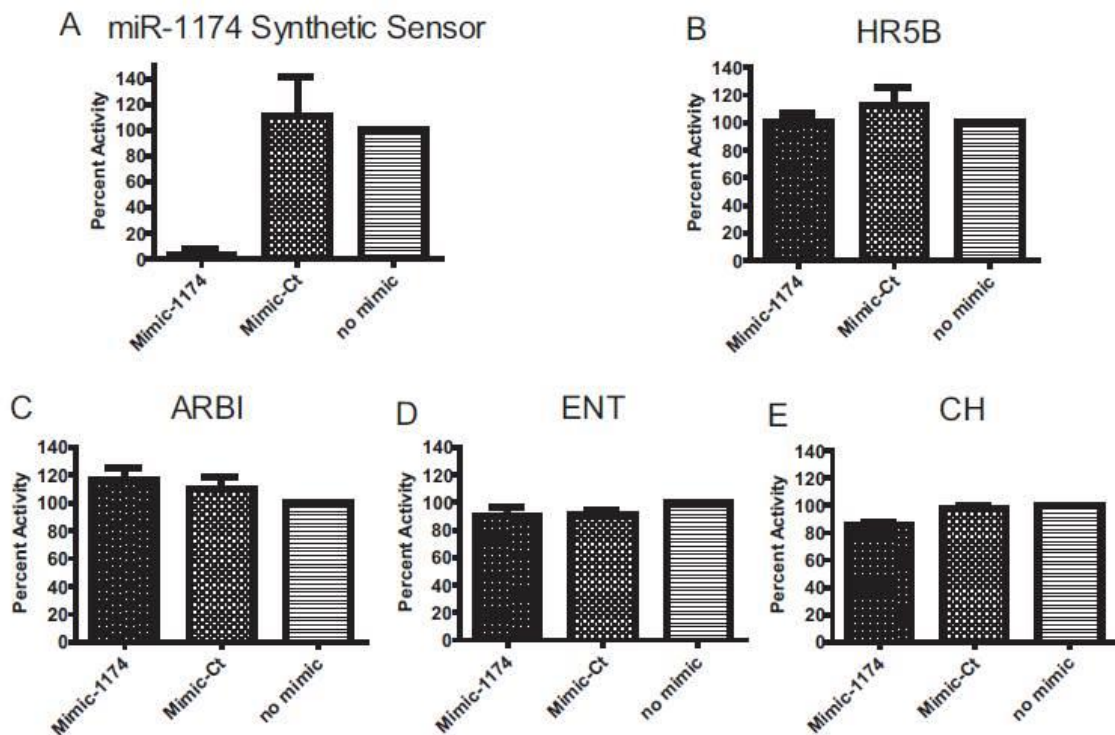
Supplemental Figure 3.1



Supplemental Figure 3.2 Caption

miR-1174 directly targets AAEL002510, Serine hydroxymethyltransferase 3' UTR *in vitro*. (A) miR-1174 synthetic sensor containing three sites complementary to miR-1174 with four nucleotide linkers between each miR-1174 binding site used as a positive control. (B) AAEL012079, Heat Repeat Containing 5B. (C) EL001779, Apoptosis-related Bax inhibitor. (D) AAEL005411, Equilibrative nucleoside transporter. (E) AAEL010558, Conserved hypothetical. Data represent the percentage activity (Δ Fold Activity $\times 100$) average $\pm$ SEM of triplicate samples.

Supplemental Figure 3.2



Supplemental Figure 3.3 Caption

Expression levels of serine hydroxymethyltransferase (SHMT) transcript and miR-1174 in different tissues of female *Ae. aegypti*. Tissues were collected from mosquitoes at 72 h posteclosion (PE) (A and B) and at 24 h PBM (C and D). qRT-PCR; error bars depict $\pm$ SEM. (E and F) miR-1174 and miR-1175 levels in females used in the experiment in Fig. 4C. qRT-PCR; error bars depict $\pm$ SEM.

Supplemental Figure 3.3

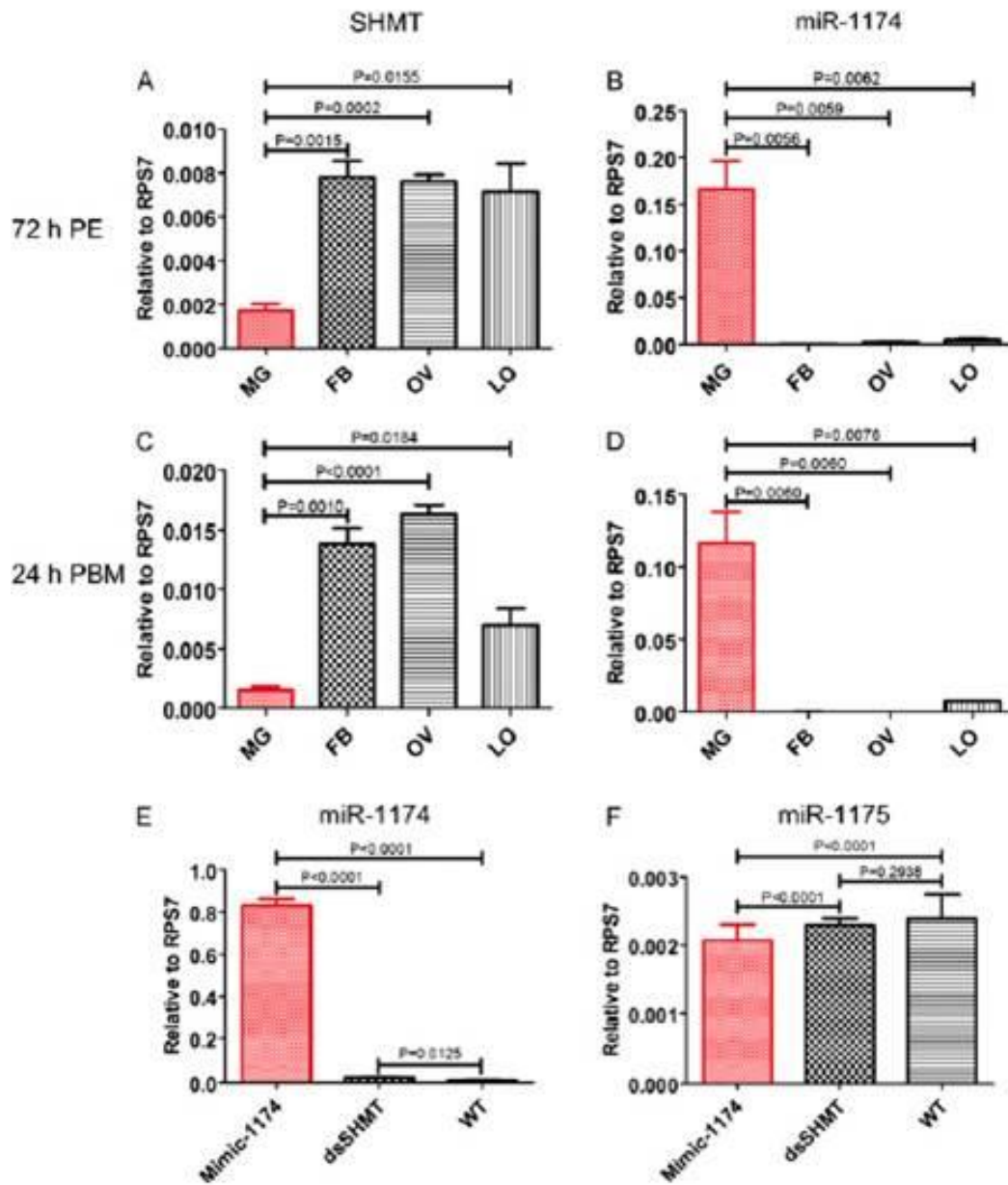


Table 3.1: Top five targets selected for dual luciferase reporter assay selection

Gene ID	Ortholog ID	Gene name	Abbreviation	Species	Programs
AAEL002510	AGAP004900	Serine hydroxymethyl transferase	SHMT	Aae	IN; TS; PITA; MR; RH
AAEL012079	AGAP002215	Heat Repeat Containing 5B	HR5B	Aae	IN; TS; PITA; MR; RH
AAEL001779	AGAP005775	Apoptosis-related Bax inhibitor	ARBI	Aga	IN; TS; PITA; MR; RH
AAEL005411	AGAP003892	Equilibrative nucleoside transporter	ENT	Aae, Aga	IN; TS; PITA; MR
AAEL010558	AGAP000964	Conserved hypothetical	CH	Aae, Aga	IN; TS; PITA; MR

Abbreviation, gene name abbreviation; Species, species in which the target was detected; Programs, programs that detected a gene target: Aae, *A. aegypti*; Aga, *A. gambiae*; IN, "in-house"; TS, TargetScan; PITA, Probability of Interaction by Target Accessibility; MR, miRanda; RH, RNAhybrid.

CHAPTER IV

Mosquito-specific MicroRNA-1890 Targets the Juvenile Hormone-regulated Serine Protease *JHA15* in the Female Mosquito Gut

4.1 Abstract

Females of the hematophagous mosquito species require a vertebrate blood meal to supply amino acids and other nutrients necessary for egg development, serving as the driving force for the spread of many vector-borne diseases in humans. Blood digestion utilizes both early and late phase serine proteases (SPs) that are differentially regulated at the transcriptional and post-transcriptional level. To uncover the regulatory complexity of SPs in the female mosquito midgut, we investigated involvement of miRNAs in regulating the juvenile hormone (JH)-controlled chymotrypsin-like SP, JHA15. In this report, we identified regulatory regions complementary to the mosquito-specific miRNA, miR-1890, within the 3' UTR of JHA15 mRNA. Expression of JHA15 is highest post eclosion and drastically declines post blood meal (PBM), coinciding with miR-1890 peak expression. Depletion of miR-1890 exhibits defects in blood digestion, ovary development and egg deposition. JHA15 mRNA and protein levels are increased in female mosquito with miR-1890 inhibition. We confirmed that JHA15 is an authentic target of miR-1890 *in vitro* via Dual Luciferase Reporter Assay and *in vivo* through JHA15 RNA interference rescue experiments in the miR-1890 depletion background. Furthermore, we found that miR-1890 is regulated by 20E signaling PBM.

4.2 Introduction

Hematophagous female mosquitoes utilize a nutrient-rich vertebrate blood meal for rapid egg development. The female mosquito gut serves as the central organ for blood digestion and an entry point for pathogen proliferation. Disease pathogens enter the female mosquito gut with ingested blood and exploit mosquitoes for obligatory stages of their life cycles. Hence, deciphering the molecular mechanisms governing blood digestion is of utmost importance for devising innovative vector and pathogen control methods.

In mosquitoes, multiple enzymes are involved in the digestion of blood. The two major classes of secreted proteases in the blood fed midgut are endoproteases, represented by trypsin-like and chymotrypsin-like serine proteases (SPs), and exopeptidases, that function as aminopeptidases and carboxypeptidases (1). Blood digestion functions in a biphasic fashion, utilizing early and late phase digestive proteases that are specifically regulated at the transcriptional and post-transcriptional level, and are thought to function in a redundant manner. In *Aedes aegypti*, the early trypsin (*AaET*) gene is expressed and its mRNA accumulated in midgut cells prior to blood feeding, while the synthesis of the AaET protein occurs soon after blood feeding (1-3). Expression of the *AaET* gene is under the control of juvenile hormone (JH) (3), while the amino acid (AA)/target of rapamycin (TOR) pathway regulates AaET translation after blood feeding (4). The gene encoding the chymotrypsin-like SP, *JHA15*, is also expressed in the Aedes midgut prior to blood feeding, and its expression is controlled by JH (5). Although its mRNA profile is similar to that of *AaET*, the JHA15 protein is readily detected in the

midgut of both non-blood fed and blood fed mosquitoes. The major events of blood digestion occur later after blood feeding. The best-studied example of a late digestive endoprotease is *Ae. aegypti* Late Trypsin (AaLT). The transcript level of the *AaLT* gene reaches maximum by 24 h post blood meal (PBM) (1, 6). It has been shown that insulin-like peptides and TOR regulate expression of the *AaLT* gene (7). Despite significant progress in understanding mosquito blood digestion and digestive enzyme regulations, the entire molecular complexity of regulatory mechanisms governing blood digestion in female mosquitoes has yet to be revealed.

MicroRNAs (miRNAs) are small 22 nt regulatory RNAs that regulate gene expression post-transcriptionally in plants and animals (8). Midgut-specific miRNAs have been identified in *An. gambiae* (9). In *Ae. aegypti*, several miRNAs exhibit stage-specific expression, and their levels are enhanced in the midgut of blood-fed females (10), implying miRNA involvement in blood-meal-associated events. The first functional study of a mosquito miRNA revealed that miR-275 is required for blood digestion and egg maturation in *Ae. aegypti* (11). More recently, the mosquito- and gut-specific miR-1174 was shown to function in sugar absorption, fluid excretion, and blood intake in the gut - further supporting miRNA function in regulating gut functions (12). To identify factors that function in the regulation of digestive SPs in the female mosquito gut, we investigated involvement of miRNAs in regulating JHA15. In this report, we identify putative miRNA binding sites within the 3' UTR of *JHA15* and examine the role of the mosquito-specific miRNA, miR-1890, in regulating JHA15 in the female mosquito midgut.

4.3 Materials and Methods

Computational miRNA target prediction

The 3' UTR of *JHA15* was retrieved from the Vectorbase database. The genebuild (AaegL1.3) of the *Ae. aegypti* genome, with well-annotated gene sequences and 3' UTR database was utilized. The *JHA15* 3' UTR was analyzed with several target prediction tools, including TargetScan (13), Probability Interaction of Target Accessibility (PITA) (14), miRANDA (15) and RNAhybrid (16). The miRNA prediction programs were used to determine if any of the 124 available *Ae. aegypti* miRNAs have a putative binding site within the 3' UTR of *JHA15*.

Cell culture and luciferase reporter assay

in vitro target validation was performed as previously described (12, 17). *Drosophila* Schneider 2 (S2) cells (Invitrogen) were kept at 28°C in Schneider's *Drosophila* medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies) and 1× Antibiotic-Antimycotic (Gibco, Life Technologies). Luciferase constructs were made by inserting the *JHA15* 3' UTR containing the putative miR-1890 binding site into the multiple cloning region located downstream of the Renilla translational stop codon within the psiCheck-2 vector (Promega). 100ng of psiCheck-2 reporters and synthetic aae-miR-1890 miScript miRNA Mimic (Qiagen) or AllStars Negative Control siRNA (Qiagen) at a final concentration of 100nm were co-transfected into *Drosophila* S2 cells using Attractene Transfection Reagent (Qiagen). A no mimic treatment was also performed. Dual Luciferase Reporter

assay was completed 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase in the psiCheck-2 Vector was used for normalization of Renilla luciferase expression. Treatments were made in triplicate, and transfections were repeated three times.

Mosquito rearing

The *Ae. aegypti* wild-type UGAL/Rockefeller strain and transgenic lines were reared at 27 °C and 80% humidity with unlimited access to 10% sugar solution and water until blood feeding, as described previously (18). Blood feeding of adult female mosquitoes was performed using White Leghorn chickens.

Antagomir and dsRNA treatments

Antagomirs were obtained from Dharmacon using the RNA module for custom single-stranded RNA synthesis. The antagomir to miR-1890 (1890Ant) was designed with the following sequence and modifications as follows: 5' mU.*.mA.*.mU.mC.mC.mA.mG.mA.mC.mC.mU.mA.mA.mU.mC.mA.mA.mA.mG.mA.mU.mU.*.mU.*.mC.*.mA.*.mG.*mC –Chl 3'. “*” is a Phosphorothioate backbone instead of the usual PO backbone. “m” is an OCH₃ group on the 2' end of the base instead of the usual OH group. A 3' cholesterol (Chl) group was added to each antagomir to enhance potency. Antagomirs were constructed as previously described (11). Mosquitoes were CO₂ anesthetized 12 h post eclosion (PE) and microinjected into the

thorax at a dose of 200 μ M in a volume of 0.25 μ L (50 pmol). Mosquitoes were allowed to recover for 3 to 4 days before blood feeding.

Double-stranded RNA (dsRNA) was produced using the MEGAscript kit (Ambion) as described previously (18). The luciferase gene was used to generate control iLuc dsRNA. After dsRNA synthesis, samples were subjected to phenol/chloroform extraction and ethanol precipitation. At 12 h PE, female mosquitoes were microinjected into the thorax with 0.5 μ g (0.25 μ L of 2 μ g/ μ L) dsRNA. For rescue experiments, mosquitoes were co-injected with 0.25 μ L of an antagomir/dsRNA mixture with a final concentration of 200 μ M antagomir and 2 μ g/ μ L dsRNA. Knockdown was confirmed via semi-quantitative PCR.

Total RNA extraction and Real-Time PCR

MicroRNA expression analysis was measured quantitatively as previously described (11, 17). TRIzol (Invitrogen) was utilized for total RNA extractions according to the manufacturer's protocol. Total RNA was treated with DNase I (Invitrogen) before cDNA synthesis using the miScript II RT Kit (Qiagen). miRNA expression was analyzed quantitatively via the miScript SYBR Green PCR kit (Qiagen) according to manufacturer's protocol. Quantitative measurements were performed in triplicate and normalized to the internal control of the housekeeping genes: S7 ribosomal protein (RPS7), actin.

Immunoblot

Protein analysis of JHA15 was done as described previously (17). Blood was removed from female mosquito midguts 24 h PBM and homogenized in lysis buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1× phosphatase inhibitor from Sigma cat # P2850, and 1× protease inhibitor from Sigma cat # P8340). Total protein was quantified using the Bio-Rad Protein Assay. 10 ug protein was boiled in LDS (4X) NuPage sample buffer (Invitrogen) with 10X sample reducing agent (Invitrogen) for 5–10 min and run on 4-12% Tris-Glycine gels (Invitrogen) before being transferred to PVDF membranes. For detection of JHA15, JHA15 antisera (5) was partially purified by affinity chromatography using Antibody Purification Kit Protein G (Abcam) and used at 1:10,000 dilution; followed by the secondary anti-Rat-HRP (Fisher Scientific) at 1:2000 dilution. For detection of Actin, beta-actin monoclonal antibody (Sigma) was used at 1:5000 dilution followed by the secondary anti-mouse-HRP (Sigma) at 1:2000 dilution.

in vitro midgut culture

The *in vitro* midgut culture was performed as previously described (19). Midguts were dissected from mosquitoes 4 days after microinjection in Aedes physiological saline (APS) buffer and incubated in a complete culture medium supplemented with amino acids and 20-hydroxyecdysone (concentration used for all experiments, 1 μ M; Sigma) for 6 h. Midguts were harvested and RNA was extracted as described above.

4.4 Results

Identification of putative miRNA binding sites within the 3' UTR of JHA15

Typically, miRNA functional analysis focuses on the characterization of a miRNA and subsequent identification of functionally relevant targets. An alternative approach for analyzing miRNA-target interaction is to first identify putative miRNA binding sites within a gene's 3' UTR region, and then functionally characterize these putative interactions. Because of the presumed role of JHA15 in blood digestion and the existing knowledge about regulation of expression, we analyzed the 3' UTR of *JHA15* (AAEL001703), encoding an early digestive protease, for the presence of putative miRNA binding sites. To identify putative miRNA binding sites within the 3' UTR of *JHA15*, we took a multi-algorithm approach using four different target-prediction programs: TargetScan (13), PITA (14), miRANDA (15) and RNAhybrid (16). Analysis with TargetScan, PITA and RNAhybrid revealed that the 3' UTR of *JHA15* contains putative binding sites for several miRNAs, including three conserved miRNAs (miR-34-3p, miR-137 and miR-315-5p) and the mosquito-specific miRNA miR-1890 (Table 1). PITA did not predict an interaction between *JHA15* and any *Ae. aegypti* miRNA. The *JHA15* 3' UTR was identified to contain a strong 7mer seed match site at positions 1-7 for miR-1890, with a putative complementary match site at positions 17-20 on the 3' end of the miRNA (Table 1). We assessed the 3' UTR of *JHA15* for its response to miR-1890 *in vitro*. The *JHA15* 3' UTR was cloned downstream of the Renilla translational stop codon within the psiCheck-2 vector to generate 3' UTR-fused luciferase reporters. When transfected into *Drosophila* S2 cells along with the miR-1890 mimic, the luciferase

reporter containing the full-length *JHA15* 3' UTR yielded 64.59% luciferase activity compared with the negative control mimic and no-mimic control samples (Fig. 1A).

Expression analysis of miR-1890 and JHA15 in the female mosquito midgut

To compare miR-1890 and *JHA15* expression, we produced a thorough time-course expression analysis of mature miR-1890 and *JHA15* in the adult female mosquito midgut by measuring relative levels of mature miR-1890 expression by quantitative real-time (qRT)-PCR analysis. We monitored the abundance of mature miR-1890 in the midgut using nine time points collected over the first reproductive cycle. We obtained total RNA samples from female mosquito midguts at 0–6, 24, 48, and 72 h PE and at 6, 12, 24, 36, and 48 h PBM. Mature miR-1890 was peaked in expression 24 h PBM and declined sharply by 36 h PBM (Fig. 1B). While miR-1890 was expressed in other tissues, such as the fat body, miR-1890 up-regulation at 24 h PBM was specific to the midgut (Fig. 1C). These results suggest that the high expression of miR-1890 in the female mosquito midgut may play an important role in tuning events associated with blood digestive processes. We also monitored the expression of *JHA15* mRNA in the midgut during the same developmental time. As previously reported, *JHA15* expression is highest PE and drastically declines PBM (5) (Fig. 1D), the latter coinciding with miR-1890 peak expression PBM (Fig. 1B). This correlation in expression profiles further suggests *JHA15* regulation by miR-1890 in the female mosquito midgut.

Depletion of miR-1890 results in misregulation of JHA15 and abnormal blood digestion

To determine the function of miR-1890 in the adult female mosquito, we inhibited miR-1890 using a sequence specific antisense oligonucleotide known as an antagomir. We designed an antagomir consisting of the reverse complement of miR-1890 (1890Ant), as well as a randomly scrambled “missense” antagomir (MsAnt) for a control. Female mosquitoes were microinjected at a dose of 50 pmol per mosquito of the 1890Ant or MsAnt at 12 h PE. To evaluate the efficiency of the miR-1890Ant, we performed qRT-PCR to monitor endogenous levels of mature miR-1890. Antagomirs designed to target miR-1890 successfully down-regulated mature miR-1890 in the female mosquito by 24 h PBM when compared to the MsAnt-treated and non-injected controls (Fig. 2A). There was no change in the relative expression levels of other miRNAs, such as mature miR-275, in the 1890Ant-treated females (Fig. 2B). Further, *JHA15* transcript and protein levels were induced in the 1890Ant-treated female midguts 24 h PBM, indicative that miR-1890 targets *JHA14* *in vivo* (Fig. 2C-D).

Female mosquitoes treated with the 1890Ant were screened for phenotypic manifestations PBM. First, we evaluated the efficiency of blood feeding and state of blood digestion at 24 h PBM. Less than 70% of the 1890Ant-treated females took blood, compared with over 90% of MsAnt-treated and non-injected females (Fig. S1A). In nearly 40% of the blood fed 1890Ant-treated females, blood remained partially undigested in the midgut (red bolus) 24 h PBM (Fig. 3A, Fig. S1B); however, at the same developmental time normal digestion (dark brown bolus) was observed in the MsAnt-treated (Fig. 3B) and non-injected (Fig. 3C) mosquitoes. Blood fed females treated with

the 1890Ant failed to fully develop mature ovaries during the first gonadotrophic cycle compared to the MsAnt-treated and non-injected controls (Fig. 3D). Ovarian follicle growth was reduced in nearly 50% of the miR-1890-depleted females compared to control mosquitoes, with an average primary follicle length of 180.71 μm (Fig. 3E, Fig. S1C). Ovaries from MsAnt-treated females were similar to those in non-injected female mosquitoes at 24 h PBM, with primary follicles reaching 218.84 μm in length on average (Fig. 3E). In addition, 1890Ant-treated females displayed reduce fecundity, laying a significantly reduced number of eggs per mosquito as compared to controls, with an average of 53.7 eggs per mosquito (Fig. 3F). MsAnt-treated females laid a similar number of eggs as non-injected female mosquitoes, with 92.2 and 99.97 eggs per female on average, respectively (Fig. 3F). Pre-vitellogenic ovary development, host seeking behavior, blood digestion and longevity were unaffected in 1890Ant-treated females, and displayed no other adverse phenotypes when compared to MsAnt-treated and non-injected controls.

JHA15 knockdown rescues miR-1890 depletion phenotype

Next, we conducted phenotypic rescue experiments through JHA15 RNA interference (RNAi) in female mosquitoes with the 1890Ant background. It was expected that the RNAi-mediated knockdown of the physiologically relevant target of miR-1890 would alleviate the adverse phenotypes caused by miR-1890 depletion. This method has proved successful in validating mosquito miRNA targets (12, 17). dsRNA designed to target *JHA15* transcripts successfully depleted *JHA15* transcript levels in female

mosquitoes (Fig. S2A). We co-injected 50 pmol of the 1890Ant or MsAnt and 0.5 μ g of JHA15 (iJHA15) or luciferase (iLuc) dsRNA into each female mosquito at 12 h PE. Females treated with the 1890Ant and luciferase dsRNA control displayed reduced follicle size and egg-deposition phenotypes characteristic of 1890Ant-treated mosquitoes, while no adverse phenotypes were observed in the MsAnt/iJHA15-treated and non-injected control mosquitoes at 24 h PBM (Fig. 4A-B). However, injection of JHA15 dsRNA and the 1890Ant partially restored the ovary-development and egg deposition in the 1890Ant/iJHA15-treated mosquitoes at 24 h PBM (Fig. 4A-C). Hence, the phenotype rescue by JHA15 RNAi in miR-1890-depleted female mosquitoes confirms that *JHA15* is an authentic target of miR-1890 *in vivo*.

miR-1890 is regulated by ecdysone signaling pathway

The mature miR-1890 levels exhibited a peak of the midgut expression at 24 h PBM and declined sharply by 36 h PBM, suggesting a possible involvement of regulatory signals activated after blood meal. To elucidate the regulatory signals involved in the transcriptional regulation of miR-1890, we investigated the effects of insulin and 20-hydroxyecdysone (20E) on mature miR-1890 levels in the midgut. Digestive systems that included midguts were isolated from 4 day old female mosquitoes and incubated *in vitro* with the presence or absence of specific factors in a culture medium. We then evaluated the level of mature miR-1890 from our *in vitro* experiments. Midguts incubated in the medium with amino acids (AAs) or medium alone showed a low, baseline level expression of miR-1890 (Fig. 5A). Insulin with AAs did not increase miR-1890 levels;

however, the miR-1890 expression rose significantly after incubation in medium containing both AAs and 20E (Fig. 5A). This indicated that 20E, but not insulin, may regulate miR-1890 levels in the mosquito midgut.

To substantiate *in vitro* experiments, we investigated the *in vivo* effect of RNAi depletions of potential factors involved in regulation of the miR-1890 in the mosquito midgut. We performed RNAi-mediated depletion of the ecdysone receptor (EcR) and insulin receptor (InR) *in vivo* (Fig.S2). The dsRNA for EcR was designed using the region common to both EcR-A and EcR-B isoforms. We injected female mosquitoes with EcR, InR or Luc dsRNA at 12 h PE and assayed the level of mature miR-1890 in EcR and InR-depleted female mosquitoes 24 h PBM. We found that miR-1890 levels are substantially decreased in the EcR-depleted females while remaining unchanged in InR RNAi-treated mosquitoes compared to the iLuc and non-injected controls (Fig. 5B, S2B-C). Thus, *in vivo* RNAi experiment results were in agreement with those from the *in vitro* tissue culture, strongly suggesting that the 20E signaling pathway controls miR-1890 expression PBM.

To further investigate the possible influence of the 20E regulatory cascade on miR-1890 in the female mosquito midgut, we performed RNAi depletions *in vivo* of two early genes of the 20E genetic hierarchy– E74B and *broad* (Br). The dsRNA for Br was designed using the region common of all Br isoforms, while E74 dsRNA was specific to the E74B isoform. We injected female mosquitoes with E74B, Br or Luc dsRNA at 12 h PE and assayed the level of mature miR-1890 at 24 h PBM. Females treated with E74B dsRNA, but not Br dsRNA, displayed a significant reduction in miR-1890 expression

compared to iLuc and non-injected controls (Fig. 5C, S2D-E). We used a pattern-search approach to identify the presence of putative E74 and Br isoform binding sites within the promoter of miR-1890. Analysis the 2 kb region upstream of the miR-1890 precursor sequence revealed the presence of several putative E74 (C/AGGAA) and Br-Z2 (TTTATCATTT) binding motifs at positions 1581-1608. Taken together, these results suggest that miR-1890 is regulated by the 20E signaling cascade, possibly through an interaction with E74B and the miR-1890 promoter.

The *JHA15* expression profile is closely correlated to the changing titers of JH in the female mosquito (Fig. 1B). Moreover, JH involvement in *JHA15* regulation is supported by *in vitro* analysis that indicates *JHA15* responsiveness to topical application of increasing doses of JHIII and the JH analog methoprene (5). To support this work, we performed RNAi depletions *in vivo* of the JH receptor, Methoprene-tolerant (Met) (Fig. S2F) (20). We injected female mosquitoes with Met or Luc dsRNA at 12 h PE and assayed the level of *JHA15* at 4 days post injection. Indeed, dsRNA mediated Met depletions in female mosquitoes resulted in a decreased expression of *JHA15* compared to iLuc and non-injected controls (Fig. 5D), further supporting *JHA15* regulation by the JH signaling cascade.

4.5 Discussion

In order to better understand the post-transcriptional regulation of SPs we search for regulatory links between mosquito gut SPs and miRNAs. Using a multi-algorithm approach, followed by *in vitro* cell culture assays, we identified a putative miR-1890

binding site within the 3' UTR of *JHA15*. *JHA15* and mature miR-1890 display opposing expression profiles; *JHA15* transcripts are highest PE and decline soon after blood feeding, while mature miR-1890 is induced PBM. Systemic depletion of miR-1890 results in increased *JHA15* transcripts and protein PBM, resulting in impaired blood digestion in the gut, followed by decreased egg development and deposition. *JHA15* RNAi in the 1890Ant background partially alleviated the ovary development and egg laying phenotypes observed in the miR-1890-depleted females. These results suggest that miR-1890 plays an important role in mosquito blood digestion by regulating *JHA15* in the female mosquito.

Transcription of *JHA15* is known to be upregulated PE by JH in the midgut of adult female mosquitoes, and its transcriptional profile of *JHA15* is similar to that of *AaET*. While the *AaET* protein is produced PBM (2, 3), the *JHA15* protein can be detected in the midgut before blood feeding (5). Presence of mature miR-1890 is induced by 20E PBM, coinciding with the decrease in *JHA15* transcripts. The nature of *JHA15* expression may indicate miR-1890 functions to fine tune *JHA15* protein levels PBM, and enhanced *JHA15* levels disrupts proper blood digestion. Digestive enzymes are believed to function redundantly and cooperatively in the female mosquito midgut; enhanced *JHA15* levels in the midgut due to miR-1890 depletion could interfere with the function of other digestive enzymes required for proper blood digestion.

Insulin-like peptides have been shown to function in the regulation of late phase trypsin-like gene expression and blood meal digestion (7). InR RNAi knockdown delays the *AaLT* gene expression and activity. Furthermore, 20E is a major regulator of

reproductive events PBM; however, its role in regulating digestive enzymes is not understood. Several insect miRNAs have been shown to be regulated by 20E signaling (21-25) and insulin signaling (26-28). Because mature miR-1890 levels are enhanced PBM, we investigated the role of insulin and 20E signaling pathways in regulating miR-1890 expression. *In vitro* midgut culture assays and RNAi experiments *in vivo* revealed miR-1890 is induced by the 20E signaling cascade but not insulin, establishing a role for the ecdysone signaling pathway in controlling mosquito gut functions PBM. These results provide insight into the transcriptional and post-transcriptional control of SPs in the female mosquito. Taken together, these results suggest that the 20E-regulated miR-1890 targets the JH-regulated *JHA15* in the female mosquito to regulate blood digestive processes.

JHA15 RNAi in the 1890Ant background partially alleviated the ovary development and egg laying phenotypes observed in the miR-1890-depleted females. Furthermore, mature miR-1890 expression is not restricted to the female mosquito midgut and can be detected at high levels in the female fat body; however, induced expression of mature miR-1890 appears to only occur in the midgut after a blood meal. Tissue- and stage-specific systems to investigate the function of miR-1890 may prove beneficial in providing spatiotemporal resolution of miR-1890 function. The Gal4-UAS system combined with the miRNA sponge loss-of-function technique has been successfully employed to understand the function of a miRNA in the female mosquito fat body PBM (17). Significant progress has been made in developing strong driver lines to investigate the tissue-specific action of a gene of interest in the midgut of *An. gambiae*

(29) and *Ae. aegypti* (19) female mosquitoes PBM. These drivers combined with the miRNA sponge transgenic method may help determine the degree to which miR-1890 contributes to the phenotypes observed from the systemic depletion of miR-1890.

Expression analysis and discovery of miRNAs in mosquitoes has revealed several mosquito-specific miRNAs, including miR-1890 (9, 10, 30-34). Phenotypic divergence among animal species may be due in part to lineage- and species-specific regulation of gene expression by miRNAs. miRNAs restricted to mosquitoes may contribute to mosquito-specific functions and, as such, have potential for contributing to the development of novel mosquito control approaches. Mosquito-specific miRNAs have been shown to play important roles in blood digestion (12) and host-pathogen interactions (35-38) in female mosquitoes. In this study, we identified that another mosquito-specific miRNA, miR-1890, targets the juvenile hormone-regulated chymotrypsin-like SP, *JHA15*, in the midgut of the female mosquito. These studies indicate that these mosquito-specific miRNAs play key roles in mosquito-specific physiological functions, including blood-meal-associated events.

4.6 Acknowledgements

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research; K.J.L., and S.R. contributed new reagents/analytic tools; K.J.L., S.R., and A.S.R. analyzed data.

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Figure 4.1 Caption

JHA15 and miR-1890 display opposing expression profiles. (A) Dual Luciferase Reporter Assay for JHA15. Data represents the percent activity (Δ Fold Activity * 100) average $\pm$ SEM of triplicate samples. Percentage shown. (B) Relative expression profile of mature miR-1890 in the female mosquito midgut. Relative expression was analyzed at the following time-points: 0-6, 24, 48 and 72 h post eclosion (PE), and 6, 12, 24, 36, 48 h post blood meal (PBM). (C) Relative expression of mature miR-1890 in midgut (MG), fat body (FB), ovary (OV) and left over tissue (LO) at 71 h PE, and 24 h PBM. (D) Relative expression profile of JHA15 in the female mosquito midgut. Relative expression was analyzed at the following time-points: 0-6, 24, 48 and 72 h post eclosion (PE), and 6, 12, 24, 36, 48 h PBM. . (B-D) Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM.

Figure 4.1

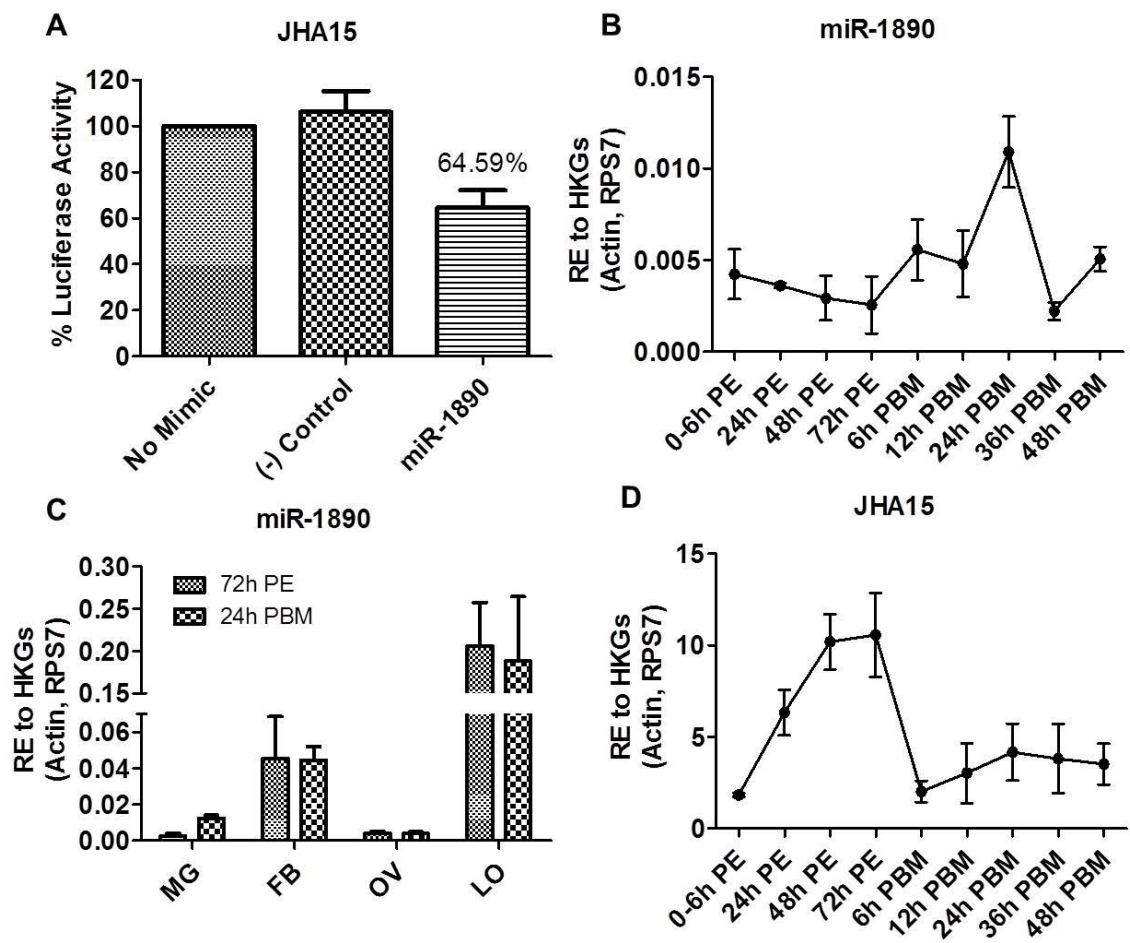


Figure 4.2 Caption

miR-1890 depletion results in increased JHA15 levels. (A) Percent relative expression of miR-1890 in the female mosquito midgut 24 h post blood meal (PBM). (B) Mature miR-275 percent relative expression in the female mosquito midgut 24 h PBM. (C) JHA15 transcript levels increases in miR-1890-depleted female mosquito midguts. (A-C) Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$. (D) Western blot analyses utilizing antibodies against JHA15 in 1890Ant-treated, MsAnt-treated and non-injected female mosquito midguts. Beta-actin was used as a loading control.

Figure 4.2

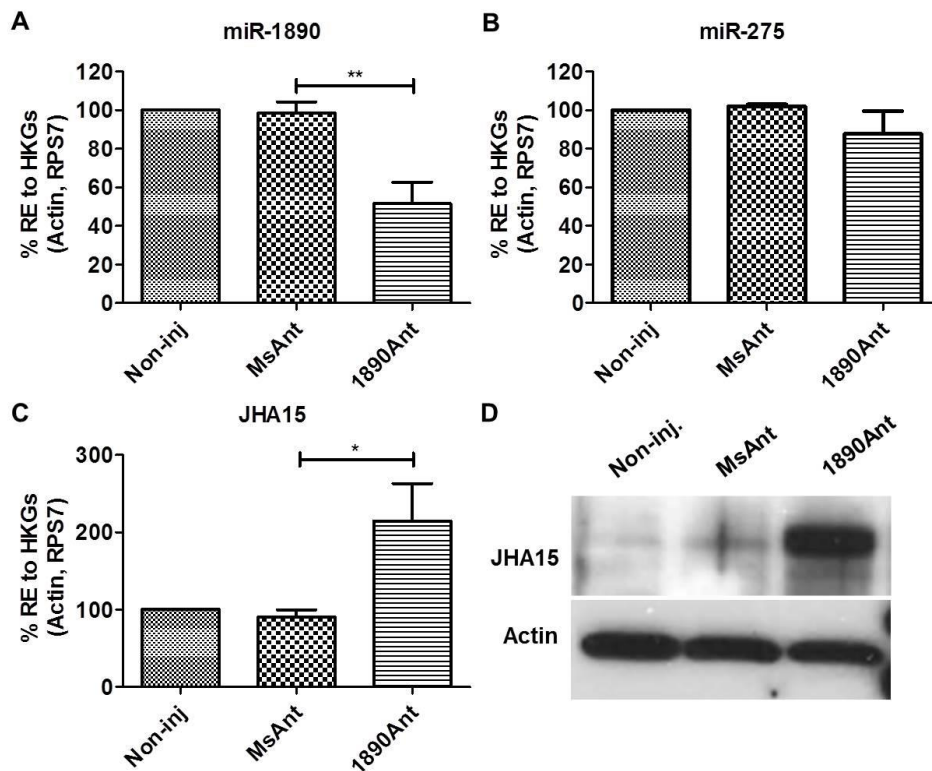


Figure 4.3 Caption

miR-1890 depletion results in impaired in blood digestion and egg development (A-C) Midguts 24 h post blood meal (PBM) of (A) miR-1890 antagomir (1890Ant)-treated, (B) MsAnt-treated, and (C) non-injected females. Images (A-C) obtained under the same conditions using the Leica M165FC stereo microscope. (D) Female mosquito ovaries 24 h PBM. Ovaries were visualized using the Leica M165FC stereo microscope. (E) Average follicle size of 1890Ant-treated, MsAnt-treated and non-injected mosquitoes 24 h PBM. Measurements were made using the Leica M165FC stereo microscope. (F) Egg numbers per female mosquito for 1890Ant-treated, MsAnt-treated and non-injected mosquitoes. (E-F) Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$.

Figure 4.3

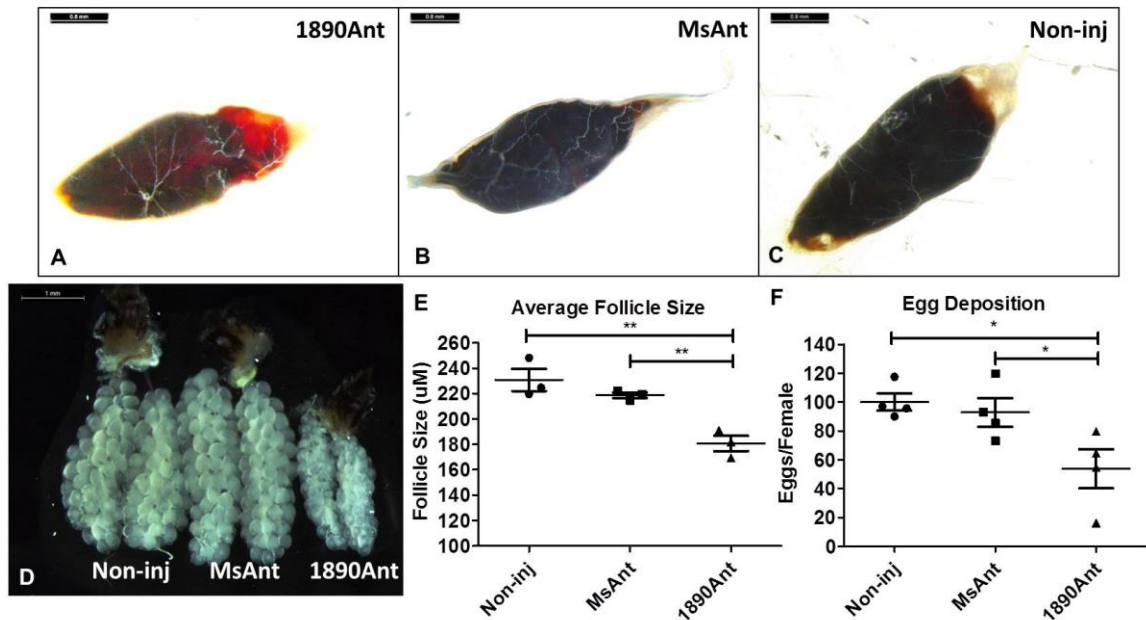


Figure 4.4 Caption

JHA15 RNAi partially rescues miR-1890 depletion phenotypes. (A) JHA15 RNAi alleviated ovary development phenotype in miR-1890 antagomir (1890Ant)-treated female mosquitoes. (B) Swim RNAi alleviated egg deposition phenotype in miR-1890Ant-treated female mosquitoes. Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$.

Figure 4.4

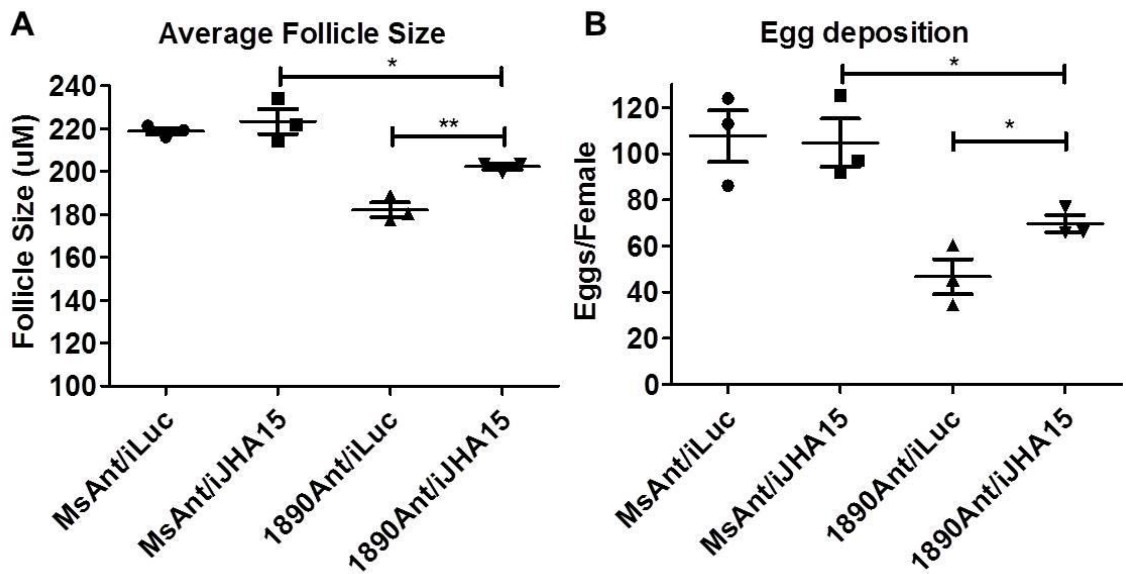
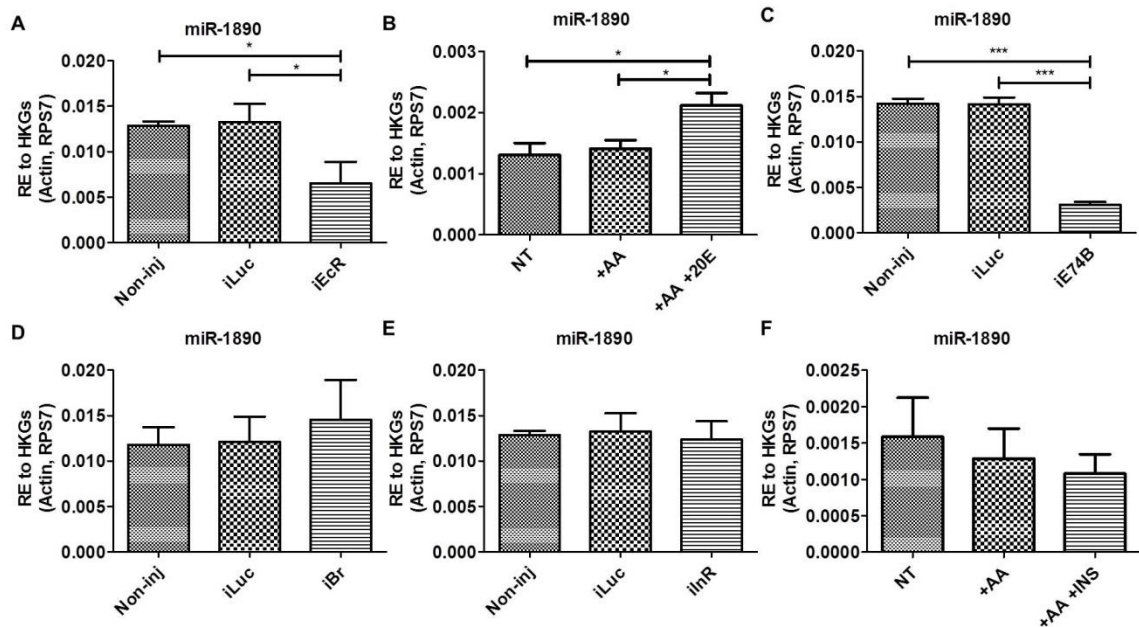


Figure 4.5 Caption

miR-1890 responds to 20E, while JHA15 is regulated by JH. (A) Mature miR-1890 expression from *in vitro* midgut. Midguts were isolated from non-blood fed female mosquitoes 72 post eclosion (PE) and incubated under indicated conditions *in vitro* for 6 h. (B) Mature miR-1890 expression *in vivo* in Ecdysone Receptor (EcR) and Insulin Receptor (iInR) RNAi treatments 24 h post blood meal (PBM). (C) Mature miR-1890 expression *in vivo* in E74B and Broad RNAi treatments PBM. (D) JHA15 expression *in vivo* in Methoprene Tolerant (iMet) RNAi-treated non-blood fed female mosquitoes. (B-D) RNAi for Luciferase (iLuc) served as a control. (A-D) Data represents three biological replicates with three technical replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4.5

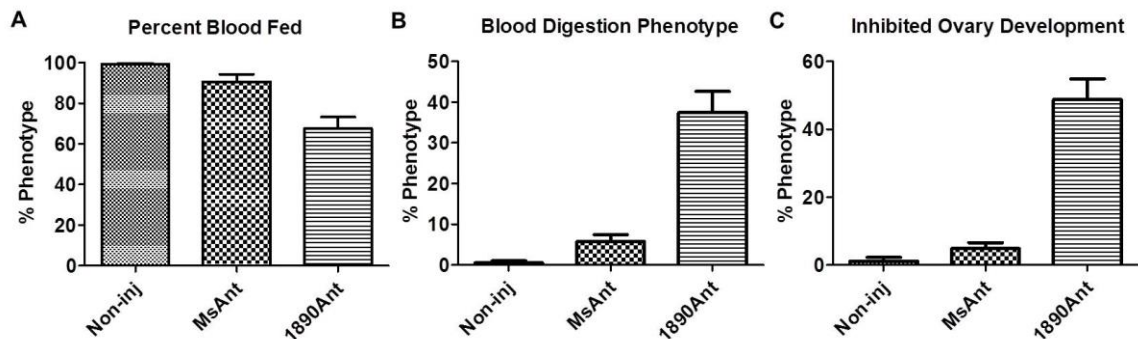


Supplemental Figure 4.1 Caption

miR-1890 depletion results in impaired in blood digestion and egg development. (A)

Percentage of female mosquitoes injected with 1890Ant that blood fed compared to MsAnt-treated and non-injected controls. (B) Percentage of female mosquitoes injected with 1890Ant displaying the blood digestion phenotype compared to MsAnt-treated and non-injected controls. (C) Percentage of female mosquitoes injected with 1890Ant displaying the ovary development phenotype compared to MsAnt-treated and non-injected controls.

Supplemental Figure 4.1



Supplemental Figure 4.2 Caption

Confirmation of gene knockdowns by dsRNA via semi-quantitative RT-PCR. (A) JHA15 (B) Ecdysone Receptor (EcR) (C) Insulin Receptor (InR) (D) E74B (E) Broad (Br) (A-E) Knockdown confirmation in female mosquitoes 24 h post blood meal. (F) Knockdown of Methoprene Tolerant (Met) in non-blood fed female mosquitoes. (A-F) RNAi for Luciferase (iLuc) served as a control. S7 Ribosomal Protein (RPS7) was used as a loading control.

Supplemental Figure 4.2

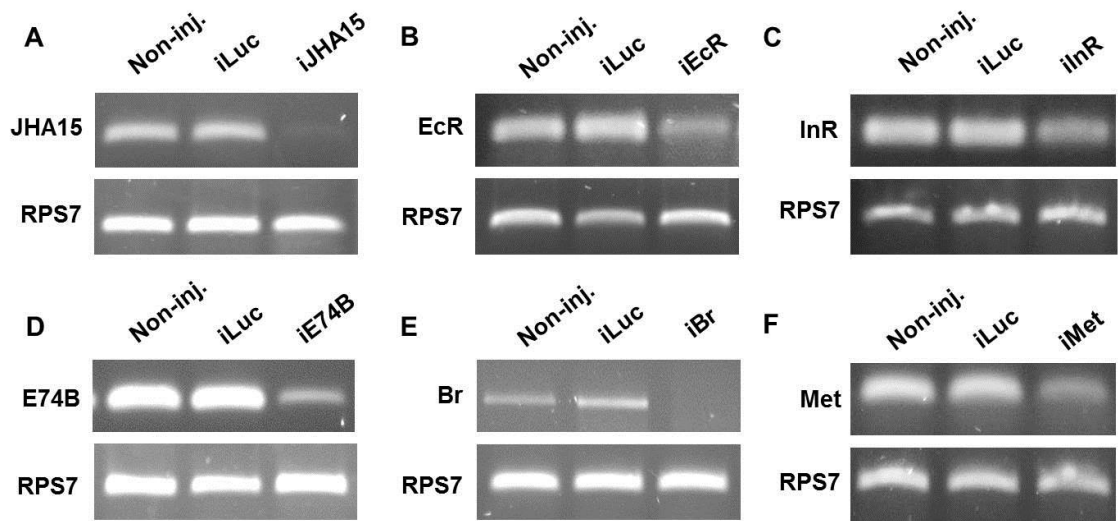


Table 4.1: Putative miRNA binding sites within the 3' UTR of JHA15

miRNA	Program ⁱ	Target Site
miR-34-3p	TS; RH	target 5' AA GGUAGG U 3' C CGUCC CACCAA miRNA 3' CCG CGCCUAU C 5'
miR-137	TS; PITA; RH	target 5' A GAAAAUAUCAAAAA C 3' GUG UCAAGCAAU CAC AGUUCGUUA miRNA 3' GAUG AUAAG U 5'
miR-315-5p	TS; RH	target 5' G AAAAU A 3' GAGUGG AUCAAAA CUCGUU UAGUUUU miRNA 3' CGAAAGA GU 5'
miR-1890	TS; RH	target 5' G AGU A 3' AGAU GAUUUCA UCUG CUAAAAGU miRNA 3' GG GAUUAGUUU 5'

ⁱ miRanda did not identify any putative miRNA binding sites in JHA15

Program Abbreviation; TS, TargetScann; PITA, Probability Interaction by Target Accessibility; RH, RNA hybrid

CHAPTER V

Conclusion of the Dissertation

5.1 Summary

Mosquito blood digestion and reproduction represent two highly regulated processes that are interconnected through the female mosquito's need for a blood meal to provide amino acids and other nutrients required for egg development. Furthermore, mosquito innate immune responses add an additional complexity to female mosquito physiology because of their capacity to serve as efficient vectors for disease pathogens. Mosquito-borne disease pathogens are acquired, and subsequently, transmitted to vertebrate hosts through consecutive blood meals. These processes are tightly controlled through a variety of gene regulatory methods, including transcriptional and post-transcriptional regulation. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level through targeting the 3' untranslated region (UTR) of messenger RNA (mRNA), resulting in inhibition of translation and mRNA decay (1). These small molecules have been shown to function in a variety of biological processes to fine-tune gene expression. Both conserved and lineage-specific miRNAs have been computationally predicted and experimentally identified in several important mosquito disease vectors (2). MicroRNAs have been shown to play important roles in regulating pathways involved in mosquito blood digestion (3), reproduction (3, 4) and mosquito-pathogen interactions (5-9).

This dissertation examines the roles of both conserved and mosquito-specific miRNAs in the regulation of blood digestion and egg development in the female

mosquito. Chapter II describes the fat body-specific role of miR-8 in regulating the Wingless signaling pathway and its function in modulating the secretory action by the fat body. Chapter III focuses on the functional characterization of the lineage-specific miRNA, miR-1174, which is found in only blood feeding mosquito species, including *Aedes aegypti* and *Anopheles gambiae*. Chapter IV investigates another mosquito-specific miRNA, miR-1890, and its regulatory role in blood digestion through targeting the digestive protease, JHA15. These studies signify the importance of miRNAs in regulating mosquito physiology, specifically important gut and fat body functions linked to reproduction.

5.2 Conserved miR-8 is essential for female mosquito reproductive processes

The miR-8/miR-200 family of miRNAs is highly conserved across divergent species, with miR-8 as the sole homolog in insects. This miRNA family is one of the most widely studied miRNA families, and has emerged as important regulators in animal development and disease (1, 10, 11). Because of its conserved nature and the medical importance of the miR-8/miR-200 family of miRNAs, miR-8 has received substantial attention in *Drosophila* studies. Studies of *Drosophila* miR-8 has implicated the miR-8/miR-200 family of miRNAs in playing a role in nervous system development and disease (12-15), development via Wingless/Wnt signaling (16, 17), growth, aging and cancer through the insulin signaling (18, 19), and development and cancer regulation by notch signaling (20).

Previously, we reported that miR-8 exhibits high levels of expression in the female mosquito fat body post blood meal (PBM) (3), suggesting that miR-8 might have a role in the regulation of female mosquito adult stages. We set out to functionally characterize miR-8 in the female mosquito fat body using both systemic and tissue-specific miRNA depletion methods. Using the Gal4-UAS system, we expressed a miR-8 sponge to achieve fat body-specific miR-8 depletion PBM *in vivo*. Systemic and fat body-specific depletions indicated that miR-8 functions in the fat body to regulate yolk protein precursor (YPP) secretion by the fat body and accumulation into the oocyte. As a consequence, females depleted in miR-8 displayed defects in ovary development and egg deposition. Furthermore, using a multi-algorithm approach for miRNA target identification, along with *in vitro* and *in vivo* target validation, we established an interaction between miR-8 and its target *Secreted Wingless-interacting molecule (Swim)* in mediating mosquito reproduction. This study provided evidence that miR-8 acts on long-range Wingless (Wg) signaling and functions in the fat body of the female mosquito by regulating the secretory action of the fat body.

Advances in mosquito biology, such as the application of the Gal4-UAS system, enhance our understanding of tissue- and stage- specific actions of genes *in vivo*. This study represents one of the first applications of the Gal4-UAS system to investigate the spatiotemporal action of a mosquito miRNA *in vivo*. In addition, we uncovered the miR-8/Wg axis as a new player in mosquito reproduction – in particular, regulating fat body secretory action. This investigation has opened a new avenue towards understanding

signaling pathways that are important for fat body function in adult insects during reproduction.

The text of Chapter I of this dissertation, in full, is a reprint of the material as is appears in Lucas et al. 2015 (21).

5.3 Mosquito-specific miR-1174 plays an important role in key gut function

While majority of mosquito miRNAs are conserved miRNAs, lineage-specific miRNAs have also been identified in mosquitoes. These miRNAs have the ability to influence phenotypic divergence among animal species with important roles in a variety of biological processes, complementing the function of conserved miRNAs (22). Studying the roles of lineage-specific miRNAs may enlighten our understanding of mosquito biology and mosquito-specific adaptations. Lineage-specific miRNAs in mosquitoes may underlie mosquito-specific events, including blood feeding and reproductive processes, and may serve as attractive targets for mosquito-specific control measures. The miR-1174/miR-1175 cluster is a lineage-specific miRNA cluster conserved in blood feeding mosquitoes. While miR-1175 is found in both blood feeding and non-blood feeding mosquitoes, miR-1174 is conserved in the blood feeding mosquito species *An. gambiae*, *An. stephensi*, and *Ae. aegypti* but not found in the non-blood feeding mosquito, *T. amboinensis* (23).

We identified the miR-1174/miR-1175 cluster was substantially upregulated in the female mosquito midgut PBM, and that expression of miR-1174 was specific to the posterior midgut. Depletion of miR-1174 in female mosquitoes resulted in defects in fluid

absorption, excretion, blood digestion and, consequently, egg development. Even prior to blood feeding, when miR-1174 depleted mosquitoes were maintained on a sugar solution/water diet, they developed enlarged crops and were unable to fly. Further, female mosquitoes that participated in a blood meal displayed a striking PBM phenotype, in which blood entered the crop and remained undigested, and, as a result, ovarian development was severely inhibited. Similar to target identification studies for miR-8, we identified *serine hydroxymethyltransferase* as a target of miR-1174 *in vivo*. We suggest that direct action of miR-1174 on the *SHMT* transcript maintains *SHMT* at a level required for normal gut functions.

With a primary role in folate metabolism, SHMT catalyzes the simultaneous conversions of L-serine to glycine and tetrahydrofolate to 5,10-methylenetetrahydrofolate in the *de novo* synthesis of deoxythymidine monophosphate (dTMP) (24, 25). dTMP serves as a precursor of deoxythymidine triphosphate (dTTP). Maintaining an optimal level of dTTP in the cell is essential for normal replication of nuclear and mitochondrial DNA (30). Furthermore, folate metabolism is regulated by iron availability, and, in humans, iron deficiency may lead to various abnormalities, including intestinal malfunction. The iron-storage protein ferritin enhances SHMT mRNA translation rate, inducing folate metabolism (26). Mosquitoes face the opposite task of removing massive amounts of iron from a blood meal to avoid its toxic effects. This is accomplished by converting iron to heme, which is rapidly excreted from the gut. Maintaining a low SHMT level in the mosquito gut is likely of critical importance to counterbalance the iron-stimulating to maintain an optimal cellular environment.

The text of Chapter III of this dissertation, in part, is a reprint of the material as is appears in Liu et al 2014 (27). The author Shiping Liu listed as the first author in that publication directed and supervised the research which forms the basis for this chapter.

5.4 Mosquito-specific miR-1890 functions in blood digestion

To identify additional miRNAs that play a key role in regulating digestive enzymes in the female mosquito midgut, we analyzed the 3' UTRs of various digestive enzymes for the presence of putative miRNA binding sites. From this analysis, we computationally predicted a putative miR-1890 binding site within the 3' UTR of the chymotrypsin-like serine protease, *JHA15*. *JHA15* is expressed in the *Aedes* midgut prior to blood feeding, and its expression is controlled by JH (28). Although its mRNA levels are reduced PBM, *JHA15* proteins are readily detected in the midgut of both non-blood fed and blood fed mosquitoes. Further, miR-1890 is a mosquito-specific miRNA expressed predominately in adult mosquitoes (29, 30). Only the mature miRNA sequence is conserved between mosquito species and extensive sequence variations occur in the remaining arm and loop of the precursor (29), suggesting recent emergence as a functional miRNA.

The *JHA15* 3' UTR responds to miR-1890 in *in vitro* luciferase assay experiments. *JHA15* transcripts and mature miR-1890 expression levels display opposing expression profiles, with miR-1890 peaking at 24 h PBM. Systemic antagomir depletion of miR-1890 results in a subtle phenotype in inhibited blood digestion, followed by a reduction in egg development and deposition. Further validating *JHA15* as a miR-1890

target, JHA15 knockdown in the miR-1890 antagomir treated backgrounds results in a restoration of normal blood digestion and ovary development. In addition, while *JHA15* was confirmed to respond to juvenile hormone, miR-1890 expression was shown to be controlled by the 20E signaling cascade. Induced by 20E, miR-1890 may function to fine tune *JHA15* protein levels PBM. Enhanced JHA15 protein levels resulting from the miR-1890 depletion disrupts proper blood digestion. Digestive enzymes function redundantly and cooperatively in the female mosquito midgut; enhanced JHA15 protein levels in the midgut could interfere with the function of other digestive enzymes required for proper blood digestion. This study provides additionally information regarding the complexity of digestive enzyme regulation.

5.5 Concluding remarks

With the advent of improved bioinformatic and genetic tools required for the study of miRNAs in mosquitoes, a greater understanding of the roles of miRNAs in these medically important insects is beginning to emerge. While miRNAs have been heavily studied in model organisms with well-established classical genetic and transgenic tools, until recently little work had been done investigating their role in non-drosophilid insects. Limited genetic tools needed to unravel the tissue- and stage-specific functions of miRNAs remains a severe hindrance in understanding the role of miRNAs in mosquito biology. The use of systemic miRNA inhibitors and mimics has been successful in identifying functions of several mosquito miRNAs; however, the newly established Gal4-UAS system in *Ae. aegypti* (31, 32) and *An. gambiae* (33) mosquitoes allows the

utilization of the miRNA sponge (34) and Tough Decoy RNA (35) inhibition technologies, or the ectopic overexpression of miRNA hairpins to evaluate spatiotemporal functions of miRNAs in mosquitoes.

Chapter II of this dissertation represents the first applications of the Gal4-UAS system combined with the miRNA sponge inhibition method to investigate the spatiotemporal action of a mosquito miRNA *in vivo*. Furthermore, a midgut-specific Gal4 driver utilizing the Carboxypeptidase gene promoter has been produced in *Ae. aegypti* for driving transgene expression in the gut PBM. Although Chapter III and IV of this dissertation identify the function of two mosquito-specific miRNAs, transgenic systems depleting these miRNAs in the female mosquito midgut may prove beneficial in understanding the tissue specific roles of miR-1174 and miR-1890. In addition, recent investigations of the CRISPR-Cas9 system in *Ae. aegypti* offers promising results that will allow genome engineering previously out of reach for non-model organisms (36-38). The CRISPR-Cas9 system has allowed precise and efficient sequence-specific miRNA inhibition (39-42). The *Aedes* CRISPR-Cas9 system may lead to gene modifications and genomic deletions of miRNA loci in *Ae. aegypti* and provide additional information on miRNA function in mosquito.

Knowledge of functions of both conserved and lineage-specific miRNAs in the female mosquito add a new layer to the understanding of complex gene networks regulating blood digestion and reproduction. Furthermore, these studies have uncovered additional pathways and regulators previously unknown to function in blood meal associated events. It is of particular importance to decipher whether miRNAs are

involved in regulation of blood digestion and reproduction, because these functions define the female mosquito's ability to transmit disease pathogens during consecutive reproductive cycles. Understanding miRNA roles in key physiological functions of vector mosquitoes may pave the way to the utilization of these small molecules in developing novel control approaches.

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APPENDIX I

Blood Meal Induced Midgut-specific Depletion of MicroRNA-8 has no Effect on Blood Digestion or Egg Development in the Female Mosquito

The binary Gal4-UAS system for *Aedes aegypti* has been recently expanded to include a driver line that allows investigation of gene function in the midgut post blood meal (1). The 5' upstream region of the Carboxypeptidase (*Cp*) gene linked to a modified yeast Gal4 protein coding sequence (Cp-Gal4) allows induction of a transgene in the midgut of female mosquitoes post blood meal (Fig. 6.1A). Development of the midgut-specific Gal4-UAS system provides a powerful tool for the characterization of regulatory mechanisms governing the expression of genes involved in blood digestion, including microRNAs (miRNAs).

The conserved miRNA, miR-8, is significantly upregulated in the blood fed midgut of female mosquitoes at 24 h PBM (2) (Fig. 6.1B). As outlined in Chapter I of this dissertation, systemic depletion of miR-8 using a sequence specific antagomir resulted in inhibited ovary development and egg deposition (3). In order to determine if miR-8 in the midgut contributed to the phenotype observed in the miR-8 depleted female mosquitoes, we utilized the midgut-specific Gal4-UAS system using the *Cp* gene promoter. Cp-Gal4/UAS-miR8-SP and Cp-Gal4/UAS-Scr-SP hybrid lines were produced by crossing homozygous responder lines with homozygous Cp-Gal4 driver lines. Genomic PCR analysis confirmed presence of both transgenes in hybrid lines (Fig. 6.1C). Hybrid transgenic lines were selected by simultaneous presence of both EGFP and dsRed eye-specific markers. Mature miR-8 levels remained unaffected in the Cp-Gal4 driver

line, Cp-Gal4/UAS-Scr-SP hybrid lines and WT females, while the Cp-Gal4/UAS-miR8-SP hybrid lines displayed a reduction of mature miR-8 expression 24 h PBM (Fig. 6.1D). Similar to controls, Cp-Gal4/UAS-miR8-SP hybrid lines with midgut specific depletion of miR-8 displayed no detectable negative effects on blood digestion, ovary development (Fig. 6.1E) or egg deposition (Fig. 6.1F). Furthermore, there were no detectable negative effects on mosquito development, pre-vitellogenic ovarian development, viability or sex ratio in the Cp-Gal4/UAS-miR8-SP hybrid lines, Cp-Gal4/UAS-Scr-SP hybrid lines, Cp-Gal4 driver or WT females.

Systemic antagomir and fat body specific depletion of miR-8 results in dramatic impairment of ovarian development and egg deposition: females displayed ovaries with drastically smaller primary follicles and fail to properly deposit eggs, suggesting impairment in signaling between the fat body and ovary (3). Fat body specific inhibition of miR-8 results in an inhibition of ovarian development and reduced fecundity. Although miR-8 has been previously shown to be significantly induced in the female mosquito midgut PBM (2), female mosquitoes with midgut specific depletion of miR-8 remained seemingly unaffected by the miR-8 knockdown with regards to blood digestion, ovary development and egg deposition. These results suggest that miR-8 in the midgut does not contribute to the miR-8 depletion ovary development phenotype, further confirming that fat body miR-8 contributes substantially for the phenotypes observed from the antagomir mediated miR-8 depletion.

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Figure 6.1 Caption

Midgut expressed miR-8 sponge has no effect on blood digestion or egg development. (A) Cp-Gal4 driver construct modified from Zhao et al. 2014. (B) Relative expression of mature miR-8 in the female mosquito midgut. Relative expression was analyzed at the following time-points: 72 h post eclosion (PE), and 24 and 48 h post blood meal (PBM). Data represents three biological replicates with three technical replicates. (C) Genomic PCR to confirm responder-driver hybrid for Cp-Gal4/UAS-miR8-SP (16-1/M4-2) and Cp-Gal4/UAS-Scr-SP lines (16-1/M3-1). (D) Mature miR-8 levels are decreased in the Cp-Gal4/UAS-miR8-SP (16-1/M4-2) female mosquitoes controls. Data represents three biological replicates with three technical replicates. (E) Average follicle size of Cp-Gal4/UAS-miR8-SP (16-1/M4-2) miR-8 depleted females compared to controls. (F) Egg numbers per female mosquito for Cp-Gal4/UAS-miR8-SP (16-1/M4-2) females compared to controls. Data represents the average of three biological replicates and are illustrated as average $\pm$ SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6.1

