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REVIEW

Spatiotemporal analysis of microRNA-8 reveals important role in mosquito reproduction

Keira J. Lucas [1,2], Alexander S. Raikhel [1,3]

[1]*Department of Entomology, University of California Riverside CA 92521, USA*
[2]*Graduate Program in Genetics, Genomics and Bioinformatics, University of California Riverside CA 92521, USA*
[3]*Institute for Integrative Genome Biology, University of California Riverside CA 92521, USA*

Correspondence: Alexander S. Raikhel
E-mail: alexander.raikhel@ucr.edu
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Female mosquitoes require a blood meal for reproduction, and their cyclic blood feeding habits result in the spread of devastating vector-borne diseases in humans. Developing a clear understanding of the mechanisms that govern the major functions linked to the female mosquito's ability to utilize blood and develop eggs is of paramount importance. Reports have indicated that microRNAs (miRNAs) are differentially expressed in various tissues of the female mosquito upon the uptake of a blood meal. In our previous research, we have reported the importance of miRNAs in regulating mosquito blood digestion through the characterization of the conserved miRNA, miR-275, and the mosquito-specific miRNA, miR-1174. Our most recent work has shown that the conserved miRNA, miR-8, targets the Wingless signaling pathway to regulate secretion of yolk protein precursors by the female mosquito fat body and accumulation into the developing ovaries. Here, we summarize the role of miRNAs in the female mosquito, particularly miR-8. We also discuss the recent advances in mosquito biology and how these genetic tools can enhance our understanding of miRNA function.

Keywords: mosquito; microRNA; Wg/Wnt; Gal4-UAS

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Introduction

Female mosquitoes serve as major vectors of many arboviral and parasitic diseases due to their need for vertebrate blood for egg production and their cyclic blood feeding habits. Vector-borne disease results in severe global health and economic burdens worldwide. *Aedes aegypti* is the major vector for many devastating arboviruses, including dengue, yellow fever and chikungunya. The global incidence of dengue virus has increased dramatically in recent years and is estimated to infect 390 million people annually [1]. *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 [2]. 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. In the female mosquito, the fat body undergoes dynamic changes in gene expression during adult development to accommodate ovarian maturation and egg production. 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 [3]. YPPs are 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 [4]. These yolk proteins provide essential nutrients vital for embryonic development. Due to the fat bodies essential role in producing and secreting YPPs necessary for egg development, it has received substantial attention in developing a better understanding of mosquito reproductive process.

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 genetic studies [5-11]. 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 [5-7]. 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 [8-10]. Further, 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 [12-15].

MicroRNAs (miRNAs) are small regulatory RNA molecules that control gene expression at the post-transcriptional level by targeting sites within the 3' untranslated region (UTR) of mRNAs [16]. miRNA-mRNA interactions typically result in translational inhibition and mRNA degradation; however, studies have identified that miRNAs can stabilize mRNA transcripts [16]. Reports have recognized these small molecules as key regulators of mosquito immunity and development [17-22]. Both infectious and non-infectious blood meals are known to alter the miRNA levels in multiple mosquito species [18, 23-27]. Various miRNAs have been shown to be differentially expressed during the vitellogenic life stage of the female mosquito [18, 23-28]. Our first analysis of a mosquito miRNA indicated that miR-275 plays an important role in the female mosquito by regulating blood digestion [18]. Depletion of miR-275 in *Ae. aegypti* females resulted in severe defects in the intake and digestion of blood. In another study, we showed that the mosquito-specific miRNA, miR-1174, is required for proper sugar absorption, fluid excretion, blood intake in the gut,

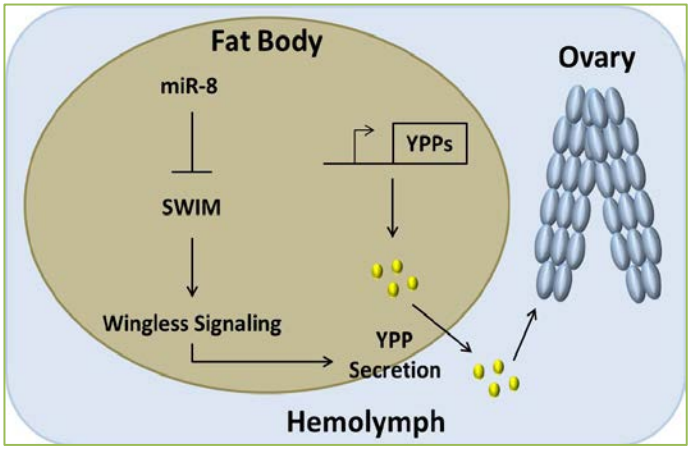

Figure 1. Schematic representation of miR-8 action in the female mosquito fat body post blood meal. miR-8 targets the Wingless signaling pathway through Secreted Wingless-interacting molecule (SWIM) to regulate Yolk Protein Precursor (YPP) secretion by the fat body and uptake into the ovary.

and, consequently, egg maturation and survival in *Ae. aegypti* and *An. gambiae* [19]. Mosquito miRNAs have the potential for influencing the development of a future generation of mosquito control measures. Furthermore, their investigation has opened a new avenue toward understanding molecular pathways important for mosquito development.

Previously we had identified that several conserved miRNAs are abundant in the female *Ae. aegypti* fat body post blood meal (PBM), including the two most abundant miRNAs - miR-275 and miR-8 [18]. The widely studied miR-8/miR-200 family of miRNAs serves as important regulators in animal development and disease. In a recent study, we reported the characterization of miR-8 and its tissue-specific role in mosquito reproductive processes [29]. We presented evidence that miR-8 functions as a regulator of Secreted Wingless-interacting molecule (Swim) and controls long-range Wg signaling activity. This interaction functions in the secretory activity of the fat body in adult female mosquitoes and plays a role in mosquito reproduction by regulating YPP secretion and uptake into the developing oocytes (Fig. 1).

We have been largely successful at identifying miRNAs that function in mosquito blood digestion and reproduction by creating a knockdown effect using an antisense oligonucleotide, known as an antagomir, that is engineered to target a specific miRNA of interest [18, 19, 29]. As an initial approach to assess the function of miR-8 in the adult female mosquito, we microinjected females with miR-8 specific antagomirs. Female mosquitoes depleted in miR-8 displayed visually striking phenotypes PBM with defects in fecundity, indicating that miR-8 plays a role in mosquito reproduction. Ovaries of miR-8 depleted females were drastically smaller

in size with a reduction in follicle size in the ovary 24 h PBM. Further, these females laid significantly fewer eggs. While this approach has been widely successful in understanding the functional roles of mosquito miRNAs *in vivo* [18, 19, 29, 30], microinjection of an antagomir creates a systemic affect without providing a clear indication of tissue specificity. The miRNA sponge transgenic method is used to dissect the function of miRNAs *in vivo* with precise spatiotemporal specificity [31]. The miRNA sponge system utilizes 8-10 consecutively arranged oligonucleotides complementary to the miRNA of interest that, when expressed, competes with the miRNA target with its miRNA binding sites. This approach represents a powerful loss-of-function technique that permits an alternative approach to classic genetic knockouts, which are typically only available for model organisms with well-established genetic tools. To elaborate on the functional properties of miR-8 in the female mosquito fat body, we applied the recently established *Aedes* Gal4-UAS system [5] to express a miR-8 specific miRNA sponge in the fat body PBM. Our fat body-specific system uses the Vitellogenin (Vg) gene promoter to drive transgene expression [5], allowing spatiotemporal genetic testing *in vivo* of miR-8 function in the female mosquito fat body PBM. Female mosquitoes with fat body-specific depletion depicted similar phenotypes as the miR-8 antagomir treated females with drastically smaller ovaries and a failure to properly deposit eggs, further confirming miR-8's role in regulating mosquito reproductive processes by the fat body. Moreover, our study is the first of its kind, in which the tissue-specific action of a miRNA was identified in mosquitoes.

We have been successful at employing a comprehensive multi-algorithm approach to identify miRNA targets [19, 29]. Through this method, we identified *Secreted Wingless (Wg)-interacting molecule* (*Swim*) as a target of *Ae. aegypti* miR-8. A recent study reported that, in *Drosophila* Swim promotes solubility of the Wg ligand and long range Wg signaling [32]. Further, the miR-8/miR-200 family of miRNAs has been confirmed to function as a regulator of the Wg/Wnt signaling pathway by targeting it at multiple levels [33, 34]. *Swim* transcript levels are increased in miR-8 depleted mosquitoes. Further, long range Wg signaling is inhibited by miR-8 depletion, consistent with findings in *Drosophila* which indicate that misregulation of *Swim* results in decreased long range Wg signaling [32]. We conducted phenotypic rescue experiments through Swim RNAi in female mosquitoes with the miR-8 depleted background. Our expectation from these experiments was that the dsRNA mediated knockdown of the physiologically relevant miR-8 target would alleviate the miR-8 depletion phenotypes. Injection of Swim dsRNA into mosquitoes with fat body specific depletion of miR-8 eased the inhibited ovary development and egg-deposition phenotypes observed in miR-8 depleted females, while restoring long range Wg signaling activity in the fat body. Along with *in vitro* Luciferase assay experiments, these results confirmed *Swim* as an authentic miR-8 target in the fat body.

Misregulation of the Wnt/Wg pathway results in reduction of adipose tissue in mammals and fat body mass in *Drosophila* [35-37]. Lipids that accumulate in developing oocytes are transported to the ovaries from the fat body by the YPPs, Lipophorin (Lp) and Vg [38]. Together with our findings, we hypothesized that improper Wg signaling, including disruption of miR-8, interferes with normal fat body functions, including the production and secretion of YPPs by the female mosquito fat body. Indeed, miR-8 depletion resulted in a drastic reduction in secretion of the YPPs, Lp and Vg, by the fat body; however, synthesis of these YPPs by the fat body remained unaffected. Further, lipid accumulation was reduced in oocytes of miR-8 depleted females. Together these results indicated that the miR-8/Wg axis is critical for the proper secretion of YPPs by the fat body and accumulation of these YPPs into developing oocytes (Fig. 1).

This work has revealed a new player regulating mosquito reproductive processes. Further investigation of the Wg signaling pathway in the adult mosquito is essential to determine the exact function of fat body Wg and its role in regulating fat body secretory action. Tissue-specific analysis for miRNA discovery and expression profiling has been performed in the mosquito midgut due to its role as the first line of defense against disease pathogens and its function in blood digestion [23, 24]. While an expression analysis of conserved miRNAs has been produced for the female mosquito fat body [18], a comprehensive miRNA discovery and expression analysis has not yet been performed for the female mosquito fat body. Such studies may shed light on additional miRNA regulators of reproductive processes in the female mosquito fat body and warrant future attention.

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