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Development and Application of the Gal4/UAS System in the Yellow Fever Mosquito *Aedes aegypti*

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of the requirements for the degree of

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by

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ABSTRACT OF THE DISSERTATION

Development and Application of the Gal4/UAS System in the Yellow Fever Mosquito *Aedes aegypti*

by

Bo Zhao

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Despite recent progress in genetic manipulations of mosquitoes, there is a much needed improvement and development of specific research tools to further facilitate research of these disease vectors. The focus of this dissertation is to extend and refine upon the Gal4/UAS system for the yellow fever mosquito, *Aedes aegypti*. The first part of the dissertation focuses on the development the blood-meal-activated, gut-specific Gal4/UAS system by utilizing a 1.1-kb, 5' upstream region of the carboxypeptidase A (CP) gene to genetically engineer the CP-Gal4 driver mosquito line. The generated driver successfully drive the expression of the effect gene followed a similar pattern as endogenous *CP* gene. Using the CP-Gal4/UAS-EGFP mosquitoes, we demonstrated that the *CP* gene is regulated by insulin and amino acids. In the second part of the dissertation, the gut-specificCP-Gal4/UAS and the fat-body specific Vg-Gal4/UAS systems were utilized in a combination with microRNA decoy to investigate the function of the, aae-miR-275 (miR-275). This approach allowed demonstrating a gut-specific

function of miR-275.Specific miR-275 silencing in the midgut resulted in dramatic defects in blood digestion and egg development in female mosquitoes. The predicted miR-275 target gene- sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-was confirmed to directly bind miR-275 by means of luciferase assay. RNA interference silencing of *SERCA* or application of SERCA-specific inhibitor Thapsigargin (TG) resulted in an apparent lack of blood digestion, inhibition of egg development, as well as diminished egg number in comparison to controls. Application of the miR-275 mimic in the CP-Gal4/UAS-miR275decoy mosquitoes restored *SERCA* transcript level and rescued the phenotype. In the last part of this thesis, I described two additional midgut-specific Gal4 driver lines that are currently under development. Together, my work provide considerable advance in developing the binary Gal4/UAS system for research of essential gene and microRNA functions in the mosquito *Ae. aegypti*.

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1.1 Insect Transgenesis

1.1.1 Transgenic technologies

Transgenesis is one of the most important technologies in modern biology. For a long time, the only way to obtain knowledge about gene function has been observation of inherited characteristics and spontaneous mutants. During the 1970s, the first chimeric animal was created by mixing embryo cells from two different mouse strains at early stages of embryogenesis to form a single embryo. The chimeric mouse that was developed from these embryonic cells exhibited characteristics of both parental strains (Brinster, 1974). Jaenisch developed the retrovirus-mediated transgenesis technique in 1976 (Jaenisch, 1976). During the 1980s, other techniques were applied to animals: DNA microinjection and embryonic stem (ES) cell-mediated gene transfer (Gordon et al., 1980; Gossler et al., 1986). Since then, rapid development of transgenic technologies has helped scientists investigate essential questions in almost all fields of biology. A milestone came in 1982, when Rubin and Spradling used a P-element-based system to successfully produce transgenic *Drosophila melanogaster*, this opened up the gate for systematic transgenesis (Rubin and Spradling, 1982). In recent years, many novel technologies have been developed, such as strategies based on zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). These new methods are revolutionizing the field of transgenesis (Wood et al., 2011; Mali et al., 2013).

1.1.2 Class II DNA Transposons

The most promising transposable elements available for insect transgenesis at present are *Hermes*, *MosI, Minos* and *piggyBac*. The common characters of these Class II DNA transposons are that they consist of inverted repeats flanking a transposase gene and function via a "cut and paste mechanism" (Atkinson et al., 2001). The size and sequence of inverted repeats are unique for a specific transposable element. The *Hermes* element, 2749 base pairs (bp) in length, is derived from *Musca domestica*. It consists of a transposase gene and that is flanked by two 17 bp inverted terminal repeats (Warren et al., 1994; O'Brochta et al., 1996). The *mariner* related element *Mos1*, isolated from *Drosophila mauritiana*, also contains a transposase gene (Jacobson et al., 1986). The *Minos* element from *Drosophila hydei* is approximately 1.8 kb in length and contains 255 bp inverted terminal repeats. *Minos* is a member of the *Tc*1 family of transposable elements (Franz and Savakis, 1991). *Hermes* and *Mos1* transposable elements have been shown to be capable of transposition in *Aedes aegypti* (Jasinskiene et al., 1998; Coates et al., 1998). *Minos* has been used as a transgene transfer vector in *Anopheles stephensi* (Catteruccia et al., 2000).

The TTAA-specific transposable element *piggyBac* was isolated from *Trichoplusia ni* (Cary et al., 1989). *piggyBac* is 2.5 kb in size, it contains 13 bp inverted terminal repeats and encodes a unique transposase enzyme (Elick et al., 1995; Elick et al., 1996). *piggyBac* transposase specifically recognizes *piggyBac* inverted terminal, catalyzes excision of the contents and integrates them at TTAA sites throughout the genome in a

relatively random pattern (Elick et al., 1995). Compared to any other insect-transposable element, the most significant characteristic of *piggyBac* transposon is that it can be excised precisely from the donor site, leaving not sign of integration (Elick et al., 1996). *piggyBac* is the most commonly used transposable element in genetic transformation of insects and transform a wide range of insect species, including the fruit fly *Drosophila melanogaster* (Handler and Harrell II, 1999), the silkworm *Bombyx mori* (Tamura et al., 2000), the housefly *Musca domestica* (Hediger et al., 2001) and the mosquito *Ae. aegypti* (Kokoza et al., 2001).

1.1.3 Transposons as Genetic Tools

To generate transgenic insects, two plasmids are necessary. In one plasmid, termed the donor plasmid, the designed transgene and a selectable transformation marker are flanked by inverted terminal repeats. Another plasmid, the helper plasmid, contains an open reading frame, which encodes the transposase enzyme under the control of an inducible promoter (for example, the regulatory region of the heat shock protein 70). The mixture of these two plasmids (in a particular ratio) is injected into the posterior region of pre-cellularized embryos. This region of the developing embryo contains the germplasm, which later forms the primordial germ cells. It is during this process that some nuclei of the germ cells will take up the two plasmids together. The transposase gene within the helper plasmid is then induced to be expressed, which excises the transgene of interest and selectable marker from the donor plasmid and integrates them into the genome, as a result some of the germline cells contain stable integration of transgenes. Surviving

adults from the injected embryos, usually called "G_0", are crossed with wild-type insects to give offspring, named "G_1". G_1 individuals are screened for the presence of the selectable marker. The individuals expressing the selectable marker are selected for further analysis.

However, only a small portion of the embryos contain the genomic transgene insertion. This is due to the fact that not every germline cell can take up both the donor and helper plasmids and finish the whole integration process. In addition the injection itself and transposase cause harm to the embryos. To solve this problem, an easily detectable transformation marker is necessary for simplifying the selection processes. A number of reliable transformation markers, such as enhanced green fluorescent protein (EGFP) and the red fluorescent protein DsRed, have been established. An artificial strong promoter - 3×P3 promoter - drives strong expression of the transformation marker in the eyes of many insects (Horn et al., 2002).

1.2 The Gal4/UAS system

1.2.1 Development of the Gal4/UAS System

Transgenic technology allows expression of a transgene under the control of a specific promoter. The selected genes regulatory regions can be used to drive particular temporal and spatial expression of transgenes. Tissue- and/or stage- specific promoters are widely used and achieve high levels of inducibility; however, the major limitation of this approach is the inability to express modified genes with potentially lethal

phenotypes. Developing the binary Gal4/UAS system has been a major step in improving the methods of genetic engineering.

Brand and Perrimon developed the Gal4/UAS system in 1993 (Brand and Perrimon, 1993). There are two major components of this system: the yeast transcription activator protein Gal4 (driver) and the upstream activating sequence (UAS) (responder), to which the Gal4 specifically binds and activates transcription. The *Gal4* gene is placed downstream of a promoter/enhancer in the driver line, while in the responder line the UAS controls expression of a target gene. The presence of Gal4 in the driver line has no effect since there is no UAS region. Crossing the Gal4 driver line and UAS responder lines produces the hybrid line, in which Gal4 binds to the UAS region and activates target gene expression in a manner that depends on the promoter/enhancer used upstream of Gal4 (Brand and Perrimon, 1993).

Due to the separation of the Gal4 driver from the UAS responder in different transgenic lines, Gal4/UAS system provides many advantages compared to other biochemical or genetic research methods. Firstly, a driver line can be crossed to numerous responder lines, harboring different transgenes, to achieve misexpression or overexpression of various genes. Secondly, generation of multiple driver lines, under the control of different promoters/enhancers, can lead to tissue- and/or stage-specific transgene expression by crossing with a responder line. Thirdly, by utilizing this system, it is possible to investigate dominant/conditional lethal genes and genes whose products have toxic effects on organism physiology.

1.2.2 Application of the Gal4/UAS System in Drosophila

The Gal4/UAS system has been essential for genetic research and used for model organisms such as *D. melanogaster*. This system provides a chance to express toxic proteins or pro-apoptotic proteins in particular cell or tissue types, which are essential for development or survival. For example, specific expression of diphtheria toxin in a non-invasive, cell-specific and cell-autonomous pattern by utilizing the Gal4/UAS system killed pioneer neurons of the embryonic central nervous system of *Drosophila* (Hidalgo and Brand, 1997).

Gain-of-function and loss-of-function are two classic strategies to study gene function. The Gal4/UAS system has been used in large-scale gain-of-function and loss-of-function genetic screens in *Drosophila*. A modified transposon, which contains UAS upstream of a basal promoter, is utilized to produce a responder line library. The integration of this modified transposon in the genome is random. When this transposon is mobilized to a genomic position, it is possible that it can place the UAS upstream of the coding sequences. By crossing a Gal4 driver with the library, coding sequences that are flanked by UAS can be transcribed, one can screen for gain-of-function phenotypes. If DNA binding domain of Gal4 is fused to a suppressor domain, this Gal4-fusion protein can have enhancer-blocking activity. By crossing this Gal4-fusion driver with responder lines from the library, phenotypes of loss-of-function can be studied (Rørth, 1996; Pascual et al., 2005).

It is possible to perform RNA interference (RNAi) *in vivo* utilizing the Gal4/UAS system. Spatiotemporal Gal4 driver lines are used to knockdown endogenous gene

expression through activating RNAi cassettes in a particular tissue or developmental stage (Kennerdell and Carthew, 2000). The Gal4/UAS system has also been used to perform tissue and stage specific microRNA loss-of-function experiments. With the help of the Gal4/UAS system, a new and powerful transgenic technology named "microRNA sponge (miR-SP)" has been performed in *D. melanogaster* to explore the function of specific microRNAs with precise spatiotemporal resolution (Loya et al., 2009).

1.2.3 Gal4/UAS System in non-drosophilid insects

The Gal4/UAS system has been established in different non-drosophilid insects such as *B. mori* (Imamura et al., 2003), *Tribolium castaneum* (Schinko et al., 2010), *Ae. egypti* (Kokoza and Raikhel, 2011) and *An. gambiae* (Amy and Gareth, 2012). In *B. mori*, by using Gal4/UAS system, overexpression of juvenile hormone-specific esterase (JHE) from the embryonic stage resulted in *premature metamorphosis* due to removal of JH. (Tan et al., 2005). Uchino et al. developed a *piggyBac*-based enhancer trap system in *B. mori* for the analysis of gene function (Uchino et al., 2008). In *T. castaneum*, it was demonstrated that both mini-Gal4 and Gal4-VP16 driven by endogenous core promoters are efficient in activating high-level transgene expression (Schinko et al., 2010).

The Gal4/UAS system has been established only for two mosquito species, *Ae. aegypti* (Kokoza and Raikhel, 2011) and *An. gambiae* (Lynd and Lycett, 2012). Kokoza and Raikhel were the first to develop the mosquito Gal4/UAS system. The driver line was transformed with the *piggyBac* transposon containing Gal4 downstream of the Vitellogenin (Vg) gene promoter (Kokoza and Raikhel, 2011). The *Vg* promoter is a fat

body and female-specific gene that is expressed only after blood feeding. This research reveals significant progress towards the development of genetic tools in mosquitoes. Recently, Lynd and Lycett have established the Gal4/UAS system in the malaria vector *An. gambiae*. Transgenic *An. gambiae* driver lines carry a mini-Gal4 gene under the control of the carboxypeptidase gene (CP) promoter. In addition, a *gypsy* insulator sequence was used the flanking transgenes in the responder construct. Crossing the driver and responder lines resulted in progeny that expressed the marker gene in the midgut. These results showed that the Gal4/UAS system can also be used to activate transgene expression in a tissue-specific manner in *An. gambiae* (Amy and Gareth, 2012).

1.3 Biogenesis and Functions of microRNAs

1.3.1 Biogenesis of microRNAs

Non-coding regions of the genome were origionally considered to be functionless DNA. Recently, the discovery of small interfering RNAs (*siRNA*), microRNAs (miRNA) and long intergenic non-coding RNAs (long ncRNAs, *lncRNA*) have proved that non-coding regions of the genome have functions that are just as important as mRNA coding regions. One of these regulatory small RNAs, known as miRNA, are RNA molecules 21-24 nucleotides in length. The first miRNA was found in *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993). Since that time, thousands of miRNAs have been identified in many organisms. In insects, the majority of miRNA loci are transcribed by RNA polymerase II to form an initial hairpin structure termed primary miRNA (pri-miRNA) (Lee et al., 2004). Pri-miRNA transcripts are processed in the nucleus by the

microprocessor complex into a 70 nt stem-loop structure called the precursor miRNA (pre-miRNA). The microprocessor complex contains the RNase III endonuclease Drosha and a double strand RNA (dsRNA)-binding partner Pasha (Lee et al., 2003; Denli et al, 2004). Then pre-miRNA is transported outside the nucleus by Exportin-5 (Exp-5), a RanGTP-dependent dsRNA-binding protein (Lund et al., 2004; Yi et al., 2003). In the cytoplasm, another RNase III enzyme, Dicer-1 (Dcr-1), cuts off the terminal loop structure, liberating a ~22 nt miRNA–miRNA* duplex (Hutvánger et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). One strand of the duplex is loaded into the RNA-induced silencing complex (RISC), while the other strand is usually degraded. Typically, the miRNA-RISC complex seeks out target mRNAs, resulting in a silence of gene expression by translational inhibition and/or mRNA degradation (Cullen, 2004; Kim, 2005). It has recently been reported that miRNAs can also lead to stabilization of mRNA or promote translation (Vasudevan, 2012). Functional binding sites of miRNAs have been identified in the 3' untranslated regions (UTR), 5'UTRs and open reading frame (ORF) of mRNAs (Lee et al., 1993; Lytle et al., 2007; Duursma et al., 2008).

1.3.2 Methods for studying microRNAs

The approaches of studying miRNA functions fall into two classes: genetic modifications for changing miRNA gene expression and targeting mature miRNA products. The widely and effective method for investigating miRNA function *in vivo* is generating a mutant for a particular miRNA gene. A number of miRNA genes have been studied by this strategy in the fly *Drosophila melanogaster* (Bushatiand Cohen, 2007;

Bushati et al., 2008). The advantage for these technologies is that they provide absolute depletion of a specific miRNA. However, this approach is only available for model organisms with well-established genetic tools. Transposon- or homologous recombination-based approaches can also alter DNA or disrupt miRNA gene function. It is clear that conditional mutagenesis, which could produce spatiotemporal gene knockdown, would be very powerful. Unlike research in mice, recombinase-mediated cassette exchange-based methods are relatively new to insect community (Choi et al., 2009).

Antisense-mediated miRNA depletion for loss-of-function analysis is widely accepted. Using chemically modified synthetic oligonucleotides to sequester miRNAs *in vivo* is simple and highly efficient (Krutzfeldt et al., 2005). Antisense oligonucleotides provides a template complementary to the miRNA-of-interest that compete with the miRNA target for binding the miRNA. But this method has limitations for spatial analysis. To address this limitation, newly designed methods, such as miRNA Tough Decoy (TuD) and miRNA sponge, are being used to express transcripts with multiple miRNA binding sites. Meanwhile, Gal4-regulated transgene expression permits robust spatiotemporal expression of TuD or miRNA sponges. A combination of antisense oligonucleotide and Gal4/UAS system has become a versatile approach for conditional or tissue- and stage-specific depletion in living organisms (Cohen, 2009; Haraguchi et al., 2009).

1.3.3 MicroRNAs in Mosquitoes

The first mosquito miRNAs discovered were from *An. gambiae*; some of them were believed to be mosquito-specific (Winter et al., 2007). Deep-sequencing coupled with genomic and bioinformatics analysis has produced expanded knowledge of mosquito miRNAs (Mead and Tu, 2008; Li et al., 2009; Skalsky et al., 2010; Gu et al., 2013). Small-scale 454 sequencing of embryo and midgut samples from *Ae. aegypti* had identified 86 distinct miRNAs. Expression profiling of miRNAs indicated that many miRNAs were induced after the uptake of a blood meal, which suggested that miRNAs play important roles in blood digestion (Li et al., 2009). Illumina sequencing of samples from an *Aedes albopictus* C7/10 cell line and blood-fed *Culex quinquefasciatus* females lead to discovery of a total 65 and 77 miRNAs, respectively. West Nile Virus (WNV) infection caused changes in the expression levels of miR-989 and miR-92, suggesting that these miRNAs are possibly involved in WNV transmission (Skalsky et al., 2010). Gu et al. reported 119 miRNAs from multiple developmental stages of *Aedes albopictus (*Gu et al., 2013*)*.

Functional analysis of mosquito miRNA was performed to investigate their precise role in various aspects of mosquito physiological activities. It has been demonstrated that the expression of aae-miR-275 (miR-275) is induced by blood feeding in the female fat body and is regulated by the steroid hormone 20-hydroxyecdysone (20E). Depletion this miRNA resulted in a severe defects in blood digestion, fluid excretion and egg development (Bryant et al., 2010). In another study, aae-miR-375 was identified to target Toll pathway components *Cactus* and *Rel1*, which are innate immune

genes involved in anti-pathogen functions (Hussainet al., 2013). Recent studies focusing on *Wolbachia*-infected *Ae. aegypti* mosquitoes have uncovered that aae-miR-2940 exhibits differential expression in response to this endosymbiotic bacterium. Two genes, metalloprotease *m41 ftsh* and DNA methyltransferase gene *AaDnmt2*, were shown to be regulated by aae-miR-2940 (Hussain et al., 2011; Zhang et al., 2013). These findings provided evidence that *Wolbachia* hijacks host miRNAs and blocks dengue virus replication in *Wolbachia*-infected mosquitoes.

1.4 Thesis Objectives and Aims

(1) The Gal4/UAS system is a widely used bi-partite system in numerous organisms. It is considered to be a powerful technique for gene over-expression, silencing or as an imaging tool. The major concern about this system in mosquitoes is that there are limited Gal4 driver lines available. In this work, I address this limitation in the mosquito *Ae. aegypti* in order to expand Gal4 driver tools and the enhance the application of the Gal4/UAS system by generating tissue-specific driver lines. Accordingly, my first objective was the development of multiple midgut-specific Gal4 drivers.

(2) To date, understanding miRNA functions in disease vectors, such as mosquitoes, is poor. Application of new genetic tools is required for miRNA functional studies. Hence, my second objective was utilization of the miRNA Tough Decoy technology in a combination with the Gal4/UAS system with the aim of deciphering miR-275 function.

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Chapter 2: Regulation of the gut-specific carboxypeptidase: a study using the binary Gal4/UAS system in the mosquito *Aedes aegypti*

2.1 Abstract

Pathogen transmission by mosquitoes is tightly linked to blood feeding, which, in turn, is required for egg development. Studies of these processes would greatly benefit from genetic methods, such as the binary Gal4/UAS system. The latter has been well established for model organisms, but its availability is limited for mosquitoes. The objective of this study was to develop the blood-meal-activated, gut-specific Gal4/UAS system for the yellow-fever mosquito *Aedes aegypti* and utilize it to investigate the regulation of gut-specific gene expression. A 1.1-kb, 5' upstream region of the carboxypeptidase A (CP) gene was used to genetically engineer the CP-Gal4 driver mosquito line. The CP-Gal4 specifically activated the Enhanced Green Fluorescent Protein (EGFP) reporter only after blood feeding in the gut of the CP-Gal4>UAS-EGFP female *Ae. aegypti*. We used this system to study the regulation of *CP* gene expression. *In vitro* treatments with either amino acids (AAs) or insulin stimulated expression of the *CP-Gal4>UAS-EGFP* transgene; no effect was observed with 20-hydroxyecdysone (20E) treatments. The transgene activation by AAs and insulin was blocked by rapamycin, the inhibitor of the Target-of-Rapamycin kinase (TOR). RNA interference (RNAi) silence of the insulin receptor (IR) reduced the expression of the *CP-Gal4>UAS-EGFP* transgene. Thus, *in vitro* and *in vivo* experiments have revealed that insulin and TOR pathways control expression of the digestive enzyme CP. In contrast, 20E, the major regulator of post-blood-meal vitellogenic events in female mosquitoes, has no role in regulating the expression of this gene. This novel CP-Gal4/UAS system permits functional testing of

midgut-specific genes that are involved in blood digestion and interaction with pathogens in *Ae. aegypti* mosquitoes.

2.2 Introduction

Female mosquitoes require vertebrate blood for egg development, and their cyclic feeding results in transmitting pathogens of numerous devastating human diseases. The yellow fever mosquito *Aedes aegypti* has become the predominant vector for the virus that causes Dengue fever, a life threatening and debilitating disease, throughout many parts of the world. Understanding of functions linked to blood feeding is essential for elucidating mechanisms of acquisition and transmission of disease pathogens. It could pave the way toward developing novel approaches for pathogen and vector control.

Significant progress has been made through genomic and post-genomic studies in mosquitoes. Genomes of three mosquito species-*Anopheles gambiae*, *Ae. aegypti* and *Culex quinquefasciatus*-have been sequenced, permitting identification of genes involved in mosquito-specific functions (Holtet al., 2002; Neneet al., 2007; Arensburger et al., 2010). However, hypotheses arising from genetic and genomic data need to be tested *in vivo* to elucidate the function of individual genes. Germ-line transformation allows introduction of such genes of interest into mosquitoes for identification of their functions. These transgenic studies in mosquitoes have been limited to direct overexpression of the gene of interest under the control of a selected promoter (Kokoza et al., 2000; Moreira et al., 2000; Adelman et al., 2008; Papathanos et al., 2009; Cho et al., 2006; Catteruccia et al., 2005; Nolan et al., 2011). Establishment of the Gal4/UAS system in model organisms has initiated a new era in elucidating gene functions (Brand and Perrimon, 1993; Ornitz et al., 1991; Scheer et al., 1999; Hartley et al., 2002; Imamura et al., 2003; Schinko et al., 2010). Essential genes, which when modified can have potentially harmful effects on

development, behavior and fertility, can be investigated using the Gal4/UAS system. However, development of the Gal4/UAS system in mosquitoes has been slow, due to the difficulties in genetic transformation of these organisms and scarcity of available promoters with tissue/cell-, sex- and stage-specific expression. Kokoza and Raikhel established the first binary Gal4/UAS system for the female-, blood-meal-induced- and fat-body-specific expression in the mosquito *Ae. aegypti* (Kokoza and Raikhel, 2011). Lynd and Lycett have reported the midgut-specific Gal4/UAS system in the malaria vector *An. gambiae* (Lynd and Lycett, 2012). However, further refinement and development of the midgut-specific Gal4/UAS system is essential for characterization of regulatory mechanisms, governing expression of genes involved in blood digestion and pathogen interaction in mosquitoes. The goal of this work is to establish the midgut-specific Gal4/UAS system in *Ae. aegypti*.

In this study, we used the 5' upstream region of the *CP* gene to establish the *Ae. aegypti* CP-Gal4 driver line. We show that the CP-Gal4 driver activates a UAS-EGFP responder in midguts of CP-Gal4>UAS-EGFP females in a blood meal dependent manner. Using this CP-Gal4/UAS system, we have investigated the regulation of transgene expression and shown it to be regulated by insulin and amino acid/TOR pathways. The development of the female midgut-specific CP-Gal4/UAS system for *Ae.aegypti* will enhance our ability to investigate genes involved in blood digestion. It will also be instrumental for studies of midgut factors that play a role in interactions with invading pathogens.

2.3 Materials and methods

2.3.1 Mosquito Rearing

The *Ae. aegypti* UGAL/Rockefeller strain and transgenic lines were reared under identical laboratory conditions (27°C and 80% humidity) and kept in cages with unlimited access to 10% sugar solution and water until blood feeding. Three- to four-day-old female mosquitoes were blood-fed on White Leghorn chickens.

2.3.2 Plasmid Construction

For construction of the pAehsp-pBac helper plasmid (Fig. 2.S1), we used the 0.66-kb5' regulatory region of the gene encoding the heat shock protein 70 (*Aehsp70*) (Isoe et al., 2007). PCR was used to amplify the *Aehsp70* fragment with genomic DNA from the *Ae. aegypti* UGAL/Rockefeller strain (wt) as template and *Aehsp70* gene-promoter-specific primers (Table 2.S3). This fragment was incorporated into the *Sac*I-blunted site of the pBΔSac (Handler et al., 1998) to create pAehsp-pBac, placing the *Aehsp70* promoter upstream of the *piggyBac* transposase gene. The cloning strategy for the CP-Gal4 driver construct was as follows. Plasmid pBluescript-AeCPA promoter (Franz et al., 2006) was excised using *Sac*I and *Bam*HI, and the resulting 1.1-kb fragment was subcloned into the *Sma*I-*BamH*I site of the pSLfa1180fa shuttle vector (Horn and Wimmer, 2000) to generate pSL-CP. Then, a 0.8-kb *BamH*I-*Xba*I fragment of the chimeric Gal4 activator, excised from the pBac[3×P3-EGFP *afm*, *Vg-Gal4*] (Kokoza and Raikhel, 2011), was assembled into pSL-CP to form pSL-CP-Gal4. Adding a 0.25-kb *Not*I-*Afl*II SV40 terminator fragment into the pSL-CP-Gal4 produced the complete CP-

Gal4 driver cassette. This resulting CP-Gal4 driver cassette was subcloned into the pBac [3×P3-EGFP *afm*] transformation vector (Horn and Wimmer, 2000) at the *Asc*I site to produce the CP-Gal4 driver construct.

2.3.3 Germ-line Transformation of *Ae. aegypti*

Plasmid DNA used in injections was purified using the EndoFree Plasmid Maxi Kit (QIAGEN, Valencia, CA). CP-Gal4 driver (0.35mg/ml) and pAehsp-pBac helper (0.25mg/ml) plasmids were re-suspended in 0.1mM phosphate buffer (pH 6.8, containing 5mM KCl) and injected into the pre-blastoderm-stage eggs. The development of CP-Gal4 driver line was performed following a previously described process (Kokoza and Raikhel, 2011). G_1 progeny were selected by monitoring the EGFP fluorescent eye marker under a Nikon SMZ800 fluorescence microscope fitted with GFP-B filter. The CP-Gal4>UAS-EGFP line was established as described previously (Kokoza and Raikhel, 2011), and the hybrid mosquitoes were selected by the presence of two eye-specific selectable markers, DsRed and EGFP. A Nikon SMZ800 fluorescence microscope fitted with DsRed and GFP-B filter sets was used for this selection.

2.3.4 Molecular Analysis

Genomic DNA was extracted from adult mosquitoes using DNeasy tissue kit (QIAGEN, Valencia, CA). In genomic PCR reactions, 200 ng genomic DNA was used under conditions following the manufacturer's protocol for the Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). Inverse PCR was performed on the M16-1 and F4-4 driver

lines to investigate the insertion location in the genome. A total of 2 μg genomic DNA was digested with *Mbo*I and *Hpa*II (NEB, Ipswich, MA), and 1 μg of digested product was self-ligated in 400-μl total volume overnight at 16°C using T4 DNA Ligase (NEB, Ipswich, MA). The ligation product was purified and dissolved in 30 μl ddH_2O. PCR was carried out to amplify DNA flanking the insertion locations using specific primers (Table 2.S3), and the products were sequenced and verified against the VectorBase sequences, using Basic Local Alignment Search Tool (BLAST).

For each experimental treatment, total RNA was extracted from 10 midguts using Trizol (Invitrogen, Carlsbad, CA). cDNAs were synthesized from 2 μg of total RNA after DNaseI (Invitrogen, Carlsbad, CA) treatment, using Omniscript Reverse transcriptase kit (QIAGEN, Valencia, CA) in 20μl of the reaction mixture. RT-PCR and qRT-PCR for selected transcripts were done and analyzed as described previously (Kokoza and Raikhel, 2011; Roy et al., 2007).

2.3.5 Immunofluorescence

10 midguts from each treatment were fixed at room temperature for 20 min in 3.7% (w/v) formaldehyde in phosphate buffered saline (PBS). Fixed midguts were washed (three times, 5 min each) in PBS-T, which contained 0.3% Triton X-100 (v/v) in PBS, before being blocked for 1 h in 3% (w/v) bovine serum albumin in PBS-T at room temperature. After rinsing in PBS-T, midguts were incubated at 4°C overnight with anti-GFP antibodies diluted 1:200 in PBS-T (polyclonal, developed in rabbit, Sigma-Aldrich, St. Louis, MO). Midguts were washed and incubated with Fluorescein-conjugated Anti-

Rabbit IgG (Vector, Burlingame, CA) diluted 1:400 in PBS-T for 2 h at room temperature, then mounted using VECTASHIELD Mounting Medium (Vector, Burlingame, CA). Images were captured and analyzed under a Leica M165 FC fluorescent stereo microscope and using LAS V4.0 software. Photographs are representative of ten mosquitoes for each treatment.

2.3.6 *In vitro* Midgut Culture

The *in vitro* organ culture was performed as previously described for the fat body (Roy et al., 2007). Tissue was incubated either in culture medium lacking AAs (AA−; containing equimolar amounts of mannitol in place of AAs) or with medium containing all the AAs (AA+) for 8 h at 27°C (1 midgut/60 µl/dish). In addition, the following reagents were used: bovine insulin at a final concentration of 17 µM (Sigma-Aldrich, St. Louis, MO, USA), 20 pmol rapamycin (LC Laboratory; dissolved in ethanol) and 20E at the final concentration of 10 µM (Sigma-Aldrich, St. Louis, MO, USA; dissolved in ethanol). Midguts were dissected from 3-day-old, non-blood-fed females.

2.3.7 RNA Interference

Double-stranded RNA (dsRNA) was produced using the MEGAscript T7 kit (Ambion, Austin, TX, USA), and a total of 2 µg in 0.3–0.5µl distilled water was injected into 1-day-old female mosquitoes. These mosquitoes were maintained on sucrose solution for 4 days, then given a blood meal, after which their midguts were isolated (at

24 h PBM). Transcript abundance was analyzed using qRT-PCR analysis as described above. RNAi depletion of luciferase (dsLuc) served as a control.

2.4 Results

2.4.1 Establishment of CP-Gal4 Transgenic Lines

piggyBac transposable element was utilized to create the transgenic *Ae. aegypti* CP-Gal4 driver plasmid (Fig. 2.1A). This CP-Gal4 driver plasmid contained the 1.1-kb 5' upstream region of the *CP* gene that was linked to a modified yeast Gal4 protein coding sequence (Kokoza and Raikhel, 2011; Kulkarni and Arnosti, 2003), followed by a SV40 polyadenylation signal. The CP-Gal4 driver cassette contained a selectable marker, consisting of the *EGFP* gene driven by the eye-specific *3×P3* gene promoter. To construct the *piggyBac* helper plasmid, we employed the 0.66-kb 5' upstream promoter region of the *Ae. aegypti* heat shock protein70 gene(*Aehsp70*) (Isoe et al., 2007) instead of the previously used *Drosophila melanogaster hsp70* gene (Handler and Harrell II, 1999) (Fig. 2.S1).

The pre-blastoderm embryos of the UGAL/Rockefeller *Ae. aegypti* strain (wt) were injected with a mixture of the CP-Gal4 driver and helper constructs. Of 1084 injected embryos, 90 survived to adulthood. These G_0 mosquitoes were separated by sex with 43 males and 47 females. Each G_0 male was out-crossed individually with 5 wt females giving rise to 43 male families (M1-M43). 47 females were separated into 6 groups. In each of these six G_0 group, females were out-crossed with an equal number of wt males, forming 6 female families (F1-F6). G_1 progeny (larvae and pupae) were

examined for the presence of EGFP expression in their eyes. Based on this criterion, two individuals were selected, M16-1 from M16 male family and F4-4 from F4 female family. Their progeny was established as homozygous based on the selection for 100% eye-specific EGFP expression of mosquitoes in each line. Pupae of the M16-1 line showed stronger eye-specific marker gene expression than those of the F4-4 line (Fig. 2.1B-C). Results indicated that the transformation efficiency was 2.2% (Table 2.S1).

Genome integration of the CP-Gal4 construct in the M16-1 and F4-4 driver lines was confirmed by means of genomic polymerase chain reaction (PCR) using specific primers for the *piggyBac* transposon left and right arms, as well as the CP-Gal4 sequence (Fig. 2.1E). The UAS-EGFP sequence was used in these PCR analyses as a control. While *piggyBac* transposon sequences (left arm and right arm) were detected in the driver and responder transgenic lines, the CP-Gal4 sequence was only present in the genomic DNA isolated from the CP-Gal4 driver lines, M16-1 and F4-4. DNA from the UAS-EGFP responder line contained UAS-EGFP responder sequence that was not present in either the driver line or wt mosquitoes (Fig. 2.1E).

Genome incorporation of the *CP-Gal4* transgene (*CPT*) was determined by means of inverse PCR analysis using genomic DNA from M16-1 and F4-4 transgenic lines. Insertion of the *piggyBac* transposon with canonical TTAA target sites containing the *CPT* was found in M16-1 and F4-4 transgenic lines (Table 2.S2). The insertion sites were located outside of predicted gene coding sequences (VectorBase AaegL 1.3) in both transgenic lines. The UAS-EGFP responder line with a DsRed selectable marker has been generated and characterized previously (Kokoza and Raikhel, 2011).

The pattern of *CPT* expression in M16-1 and F4-4 transgenic lines was investigated using RNA isolated from the digestive system (midgut) of female mosquitoes from the wt, UAS-EGFP responder, M16-1 and F4-4 transgenic driver lines at 72 h post-eclosion, a time point during the previtellogenic (PV) stage before blood feeding and several time points after blood feeding (Fig. 2.2A-D). This analysis was accomplished by means of the reverse-transcriptase polymerase chain reaction (RT-PCR), using primers specific to the endogenous *CP,* transgenic *CPT* and *UAS-EGFP* (Table 2.S3). There was no expression of the *UAS-EGFP* transgene in midguts of any of the tested mosquito lines (Fig. 2.2A-D). The expression pattern of the *CP* gene was similar in all four tested mosquito lines; the CP transcript was barely visible before blood feeding, it increased PBM, reaching the peak at 24 h PBM, and decreased thereafter (Fig. 2.2A-D). In the M16-1 line, *CPT* expression followed a temporal pattern similar to that of the endogenous *CP* gene (Fig. 2.2C). However, in the F4-4 line, the *CPT* was expressed during the previtellogenic stage and peaked at 12 h PBM (Fig. 2.2D). Because the *CPT* expression pattern in the F4-4 line did not match that of the endogenous *CP*, the M16-1 CP-Gal4 driver line was chosen for further analysis. Expression of *CPT* was not observed in the wt or the UAS-EGFP responder lines.

2.4.2 Development of CP-Gal4>UAS-EGFP Hybrid Mosquitoes

The CP-Gal4>UAS-EGFP hybrid mosquitoes were produced by crossing the M16-1 CP-Gal4 homozygous driver line with the UAS-EGFP homozygous responder line. TheseCP-Gal4>UAS-EGFP hybrid mosquitoes exhibited the presence of both EGFP

and DsRed eye-specific markers (Fig. 2.3A-D). Genomic PCR analysis confirmed the presence of *piggyBac* transposon left arm, right arm, CP-Gal4 driver and UAS-EGFP responder sequences in the hybrid mosquitoes (Fig. 2.3E).

In the CP-Gal4>UAS-EGFP hybrid female mosquitoes, the *EGFP* transgene expression followed a pattern similar to that of the endogenous *CP* and *CPT* (Fig. 2.4A). Expression of the *UAS-EGFP* transcript was midgut-specific and was not found in the fat body (FB), ovaries (OV) or Malpighian tubules (MT) of hybrid female mosquitoes24 h PBM (Fig. 2.4B). Males from this hybrid line did not express *UAS-EGFP* (Fig. 2.4B). These data indicated that, in the CP-Gal4>UAS-EGFP mosquitoes, the *UAS-EGFP* transgene exhibited a tissue-, sex-, and stage-specific expression pattern similar to that of the endogenous *CP* gene.

To assess the EGFP protein level in the CP-Gal4>UAS-EGFP mosquitoes, we dissected and examined midgut through a fluorescence microscope. This analysis showed a strong signal in midguts of CP-Gal4>UAS-EGFP female mosquitoes at 24 h PBM. In contrast, only background signal was observed in midguts from the CP-Gal4 driver, UAS-EGFP responder lines and wt mosquitoes examined at the same time (Fig. 2.4C). This confirmed that the EGFP protein was produced in the CP-Gal4>UAS-EGFP female mosquitoes only after blood-meal activation.

2.4.3 Regulation of Transgene Expression

To elucidate the signals involved in regulation of *CP-Gal4>UAS-EGFP* transgene expression, we first used an *in vitro* organ culture assay (Fig. 2.5). Digestive systems

(midgut) isolated from the CP-Gal4>UAS-EGFP transgenic female mosquitoes were incubated in the presence or absence of specific factors in a culture medium. Results were evaluated by means of quantitative reverse-transcriptase PCR (qRT-PCR) using primers for the *CP-Gal4* (Gal4) and *UAS-EGFP* (EGFP) transgenes (Table 2.S3). The tissue incubated in the medium without AAs showed a low, baseline level expression of the *CP-Gal4>UAS-EGFP* transgene transcript, while addition of AAs to the medium resulted in a statistically significant increase in transcript levels (Fig. 2.5A). Insulin alone did not increase the *CP-Gal4>UAS-EGFP* transgene transcript level; however, the transcript level rose significantly after incubation in medium containing both AAs and insulin together. Testing expression of both the *CP-Gal4* and the *UAS-EGFP* transgene has permitted us to demonstrate that their expression is clearly co-regulated.

When we examined the EGFP protein level using immunocytochemistry, we observed a substantial increase in the signal only after incubation of midguts from CP-Gal4>UAS-EGFP hybrid female mosquitoes in the medium supplemented with AAs and insulin (Fig. 2.5B). Thus, *in vitro* organ culture experiments have shown that both insulin and AAs are required for transcription and translation of the CP-Gal4-driven EGFP in the midgut of the transgenic mosquitoes. Moreover, they exhibit a strong additive effect.

To substantiate *in vitro* experiments, we investigated the *in vivo* effect of RNAi depletions of potential factors involved in regulation of the *CP-Gal4>UAS-EGFP* transgene in the mosquito midgut. Because insulin has been identified as a principal regulator of the transgene expression, we examined the effect of RNAi depletion of the InR. There is a single gene encoding InR in the *Ae. aegypti* genome (Brown et al., 2008).

RNAi depletion of InR *in vivo* resulted in a significant decrease of the *CP-Gal4>UAS-EGFP* transgene transcript level in midguts at 24h PBM, as indicated by testing with Gal4 and EGFP probes (Fig. 2.6A). To confirm that the effect observed after InR RNAi depletions correlated with the responsiveness to insulin, we incubated midguts isolated from the non-blood fed *CP-Gal4>UAS-EGFP* transgenic female mosquitoes at 72 h post-eclosion treated with Luciferase double-stranded RNA (dsLuc) or double-stranded insulin receptor RNA (dsInR) in the amino acid-containing culture media in the presence or absence of insulin (Fig. 2.6B-C). Addition of insulin elevated the *CP-Gal4>UAS-EGFP* transgene transcript in the dsLuc-treated mosquitoes. In contrast, no response to insulin was observed in dsInR-treated mosquitoes (Fig. 2.6B-C). Thus, *in vivo* RNAi experiment results were in agreement with those from the *in vitro* tissue culture, strongly suggesting that insulin plays a major role in regulating *CP* gene expression.

Target of Rapamycin kinase (TOR) is a conserved nutritional sensor that mediates both insulin and AA signaling (Grewal, 2008). We tested whether TOR inhibition affects the expression of the *CP-Gal4>UAS-EGFP* transgene transcript. We injected 3-day-old, *CP-Gal4>UAS-EGFP* female mosquitoes with the TOR inhibitor rapamycin at different concentrations and examined at 24 h PBM. Results indicated that the transgene transcript expression was inhibited by rapamycin in a dose-dependent manner (Fig. 2.S2A), and rapamycin did not affect survival of mosquitoes (Fig. 2.S2B). Rapamycin significantly decreased the transcript level of this gene in the *CP-Gal4>UAS-EGFP* female mosquitoes treated with dsLuc (Fig. 2.7A). RNAi depletion of InR significantly decreased the level of the *CP-Gal4>UAS-EGFP* transgene transcript when compared

with that of the dsLuc. However, this transcript level was completely diminished in transgenic mosquitoes co-treated with rapamycin and dsInR (Fig. 2.7A). Rapamycin also prevented elevation of this transcript by insulin in the mosquito digestive system in an i*n vitro* culture assay (Fig. 2.7B).

20E is a major regulator of the PBM reproductive events; however, its role in regulating digestive enzymes is not understood. To investigate the possible influence of the ecdysone regulatory cascade on the *CP-Gal4>UAS-EGFP* transgene expression, we first investigated the effect of 20E using an *in vitro* organ culture assay, as described above. Supplementing the medium with 20E did not elevate its level, regardless of the presence or absence of AAs (Fig. 2.5A). Next, we used *in vivo* ecdysone receptor (EcR) RNAi depletion. The double-stranded RNA for EcR (dsEcR) was designed using the region common to both EcR-A and EcR-B isoforms. This depletion had no effect on the transgene transcript level, which was similar to that of the dsLuc control (Fig. 2.8). The dsEcR treatments also had no effect on the transcript abundance of the endogenous *CP* gene examined in parallel with that of the *CP-Gal4>UAS-EGFP* transgene transcript at 24 h and 36 h PBM *in vivo* (Fig. 2.8).

Analysis of the *CP* gene promoter has revealed the presence of a putative binding site—tgtttag—for Broad isoform Z4 (Br-Z4). *Br* is a downstream gene in the 20E hierarchy, although it is reportedly not entirely dependent on 20E signaling and can act independently (Brennan et al., 2001; Gancz et al., 2011). In our experiments, EcR RNAi depletion significantly reduced the level of Br-Z4 transcript, suggesting that this gene is under the control of 20E hierarchy (Fig. 2.8B). However, to further investigate a possible

involvement of Br-Z4 in regulation of the *CP-Gal4>UAS-EGFP* transgene, we conducted Br-Z4 RNAi depletion. The double-stranded RNA of Br-Z4 (dsBr-Z4) treatment did not affect the transcript abundance of the transgene gene examined at 24 h and 36 h PBM *in vivo* (Fig. 2.8C). It is possible that Br-Z4 plays a role in regulation of *CP* gene expression during other stages of the mosquito life cycle. Effects of RNAi depletions of tested factors on their own transcript levels are presented in Figure S2.

Thus, our study using the *CP-Gal4>UAS-EGFP* transgene has shown that insulin and AA/TOR signaling pathways, but not 20E, are involved in regulating the expression of the gene encoding the late-phase blood digestive enzyme CP.

2.5 Discussion

In this study, we expanded the development of the binary Gal4/UAS system for the yellow fever mosquito *Ae. aegypti* and used the 5' upstream region of the *CP* gene, which is induced by a blood meal in the midgut of female mosquitoes (Isoe et al, 2009a; Edwards et al., 2000), to genetically engineer the transgenic CP-Gal4 driver line. After a blood meal, the *CP* gene expression and enzymatic activity of CP increases rapidly and reaches its peak at 24 h (Edwards et al., 2000; Noriega et al., 2002). The 1.1-kb 5' upstream region of the *CP* gene is sufficient to lead transgene expression with a profile similar to that of the endogenous *CP* gene (Franz et al., 2006). The important feature of this driver is that the *CPT* exhibits specific expression in the female mosquito midgut, and is activated by blood feeding. We generated two *Ae. aegypti* CP-Gal4 driver lines. However, only the M16-1 line expressed the transgene in a manner consistent with that of

the endogenous *CP*, from which the regulatory DNA was derived. In contrast, the F4-4 line showed a different transgene expression profile, in which the *CPT* transcript was expressed prior to blood-meal activation and reached its peak at 12 h PBM rather than 24 h. The integration transgene site for F4-4 located 8kb upstream of a predicted gene, AAEL004671, which encodes (s)-2-hydroxy-acid oxidase. The expression pattern in the F4-4 line was possibly affected by its integration location. Transgene miss-expression has previously been reported for midgut-specific transgenes in *An. gambiae* and *Ae. aegypti* (Lynd and Lycett, 2012; Franz et al., 2011). Integration of a transgene in a specific site in the genome is critical for its correct expression. For example, integration near a certain transcription enhancer or repressor could lead to an unintended transgene expression pattern (Wallrath and Elgin, 1995), and integration near a transcription repressor or heterochromatic region could result in low levels of transgene expression (O'Kane and Gehring, 1987). In some transposable-element-generated mosquito lines, transgenes have reportedly been poorly expressed because of the position effect (Nimmo et al., 2006; Labbe et al., 2010). Franz et al. described that when the *CP* gene promoter region was used to drive EGFP reporter expression in *Ae. aegypti*, only two lines from a total number of nine transgenic mosquitoes lines robustly expressed the EGFP reporter (Franz et al., 2011). Lycett et al. (2012) found that the alpha-tubulin-1b gene promoter directed transgene expression in the head, ventral nerve cord and testes of one *An. gambiae* transgenic line; however, expression of the same transgene was observed in larval and adult muscles, fat body, cuticle and midgut secretory cells of a different transgenic line.

The lack of more advanced techniques for mosquito transformation similar to those of Drosophila complicates a challenging task of mosquito genetic transformation.

Multiple enzymes are involved in blood digestion in the midgut of female mosquitoes. These include trypsin-like and chymotrypsin-like serine endoproteases, amino- and carboxypeptidases (Isoe et al., 2009a; Isoe et al., 2009b). In *Ae. aegypti*, mRNA of the early trypsin (AaET) gene is expressed and accumulated in the midgut cells prior to blood feeding, while the synthesis of the AaET protein occurs just after blood feeding (Noriega et al., 1996). The transcript and protein levels of a digestive endoprotease, late trypsin (AaLT), reach their peaks by 24 h post blood meal (PBM) (Isoe et al., 2009b; Barillas-Mury et al., 1993). Recent studies have revealed seven additional midgut serine proteases (AaSPs); the expression profiles of genes encoding these proteases are similar to that of the *AaLT* gene (Isoe et al., 2009a; Brackney et al., 2010). The RNAi and enzymatic analyses have demonstrated differential roles of midgut serine proteases. Knockdown of AaSPVI caused a 78% decrease in the late-phase, trypsin-like activity, while knockdowns of AaLT, AaSPVI and AaSPVII inhibited degradation of the serum albumin (Isoe et al., 2009a). Carboxypeptidases represent another important group of mosquito gut proteases (Isoe et al, 2009b). The gene encoding *Ae. aegypti* carboxypeptidase A has an expression profile similar to that of the *AaLT* gene (Edwards et al., 2000). However, the regulation of the *CP* gene has not been studied.

Previous studies on amino acids suggest that these blood components serve as nutritional signal in digestive protease introduction (Caroci and Noriega, 2003). Amino acids are key stimuli, which play a role in regulating the translation of AaET (Brandon et

al., 2008). Eight insulin-like peptides (ILPs) have been reported in *Ae. aegypti*, among which AaILP3 binds the InR (Riehle et al., 2006; Wen et al., 2010). Gulia-Nuss et al. reported that amino acids and insulin signaling pathways regulate *Ae. aegypti* late phase gene expression, blood digestion and egg maturation (Gulia-Nuss et al., 2011). InR RNAi knockdown or TOR inhibition reduces the *AaLT* gene expression and the activity of this enzyme (Gulia-Nuss et al., 2011).

By using the *Ae. aegypti* CP-Gal4>UAS-EGFP line with the M16-1 Gal4 driver, we established that the expression of the *UAS-EGFP* transgene matched that of the endogenous *CP* gene as well as that of the *CPT*. *In vitro* organ-culture experiments demonstrated a synergistic action of insulin and AAs in activating expression of the *CP-Gal4>UAS-EGFP* transgene. These observations were in agreement with *in vivo* RNAi depletion experiments. RNAi silencing of InR resulted in a significant decrease in the *CP-Gal4>UAS-EGFP* transgene transcript level. Application of rapamycin, a specific inhibitor of TOR, had a strong inhibitory effect on the *CP-Gal4>UAS-EGFP* transgene transcript expression, indicating importance of the TOR kinase in regulation of the *CP* gene. Moreover, the transcript level was completely diminished in transgenic mosquitoes co-treated with rapamycin and dsInR. This inhibition was significantly stronger than that caused by InR RNAi depletion alone, suggesting additive effects of TOR and insulin. Treatment with rapamycin also prevented elevation of this transcript after incubation with either AAs or insulin *in vitro*, confirming independent effects of these regulators. The TOR kinase pathway has been reported to integrate extracellular signals such as insulin and AAs (Nave et al. 1999; Sarbassov et al. 2005). Thus, insulin and AA/TOR signaling

pathways are involved in regulating expression of the *CP* gene. Taken together, our investigation along with previous studies has revealed important roles of insulin and nutrition-driven AA/TOR pathways in regulating the expression of late-phase digestive enzymes in the mosquito midgut.

20E is the major regulator of vitellogenic events in female mosquitoes (Raikhel et al., 2005). It regulates expression of genes encoding yolk protein genes in the fat body. However, our experiments revealed no apparent role of this hormone in controlling the *CP* gene in the female mosquito midgut. The level of the *CP-Gal4>UAS-EGFP* transgene transcript was not affected as a result of *in vitro* midgut incubations in the presence of 20E. Moreover, EcR RNAi silencing had no effect on the transcript level of this transgene. The absence of ecdysone response elements (EcRE) or binding sites for any other known 20E downstream factors (other than that of BrZ4) within the *CP* gene promoter further corroborates these results. RNAi silencing has shown that BrZ4 is not involved in regulation of the *CP* gene in the gut of female mosquitoes. Thus, the presence of the BrZ4 recognition site in the *CP* promoter could be explained by a possible contribution of this transcription factor in control of *CP* gene expression during other stages of development.

In conclusion, this study contributes toward the improvement of genetics tools for mosquitoes. We developed an *Ae. aegypti* CP-Gal4 driver line that is capable of directing a female, midgut-specific and blood-meal-induced expression of the UAS/target transgene. Using the CP-Gal4>UAS-EGFP system, we have demonstrated that activation of the *CP* gene depends on insulin/InR and nutrient-driven AA/TOR pathways. In

contrast, 20E, which is the major regulator of mosquito vitellogenesis, plays no role in *CP* gene activation. Establishment of the *Ae. aegypti* CP-Gal4/UAS system permits functional testing of various midgut-specific genes involved in blood digestion and pathogen interaction.

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2.7 Figures and Tables

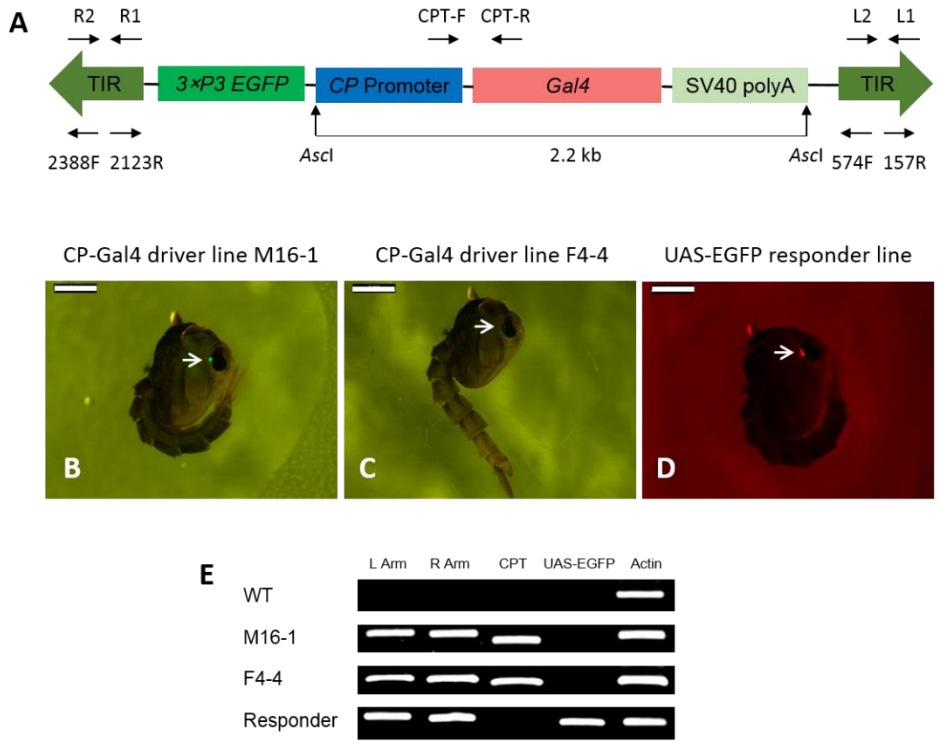

Figure 2.1. Germline transformation and molecular characterization of *Ae.aegypti* CP-Gal4 lines. (A) Schematic representation of the CP-Gal4 cassette. The CP-Gal4 cassette consists of the *piggyBac* vector plasmid containing the 5' regulatory region of the CP gene placed upstream of the modified *Gal4* and the *EGFP* marker gene under the control of the eye-specific *3×P3* gene promoter. Arrows above and below the A diagram represent the transgene-specific primers used for genomic PCR, RT-PCR and inverse PCR. (B-D) Eye-specific expression of the selectable markers, EGFP and DsRed, in *Ae. aegypti* transgenic mosquito lines. Transgenic mosquitoes were selected based on the presence of EGFP marker in CP-Gal4 lines and DsRed marker in the UAS-EGFP line at the pupal stage (arrows). Screening was carried out using a Nikon SMZ800 fluorescence microscope with GFP-B and DsRed filters, respectively. Scale bar: 1mm. (E) Genomic PCR analysis demonstrating integration of CP-Gal4 and UAS-EGFP constructs into the *Ae. aegypti* genome. Wild type (WT), CP-Gal4 driver line M16-1 (M16-1), CP-Gal4 driver line F4-4 (F4-4) and UAS-EGFP responder line (Responder), were checked. Primers were specific to the left and right arms of the piggyBac vector, left arm (L Arm), right arm (R Arm), the *CP-Gal4* transgene (CPT) and the *UAS-EGFP* transgene (UAS-EGFP). Primers for actin were used as control (Actin).

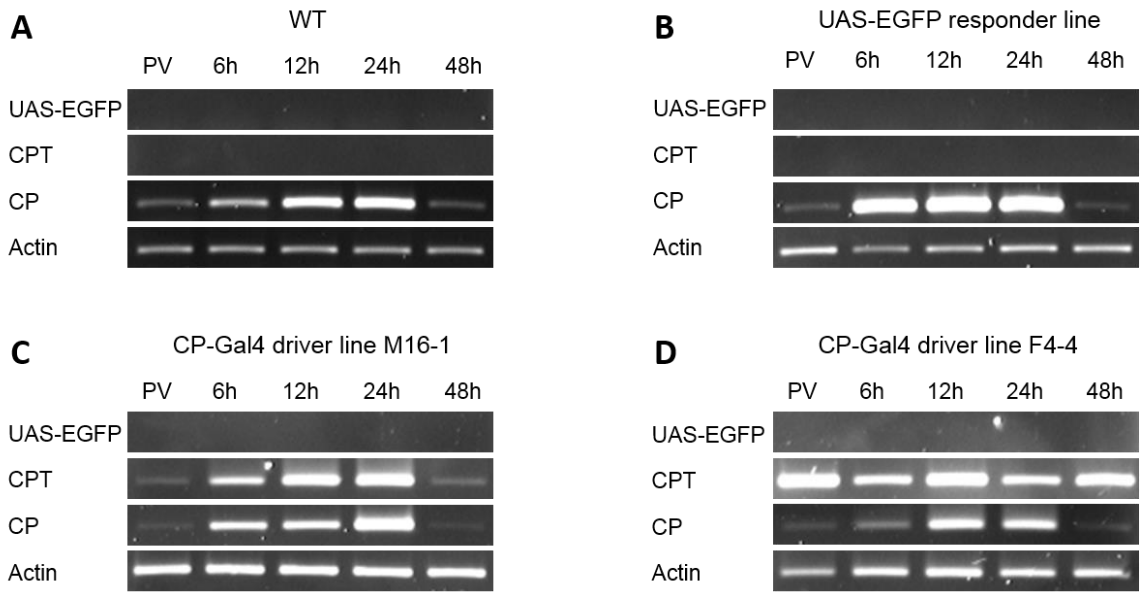

Figure 2.2. RT-PCR analysis of the *CP-Gal4* transgene expression in *Ae. aegypti* females.(A) WT, (B) UAS-EGFP responder line, (C) M16-1 CP-Gal4 driver line, (D) F4-4 CP-Gal4 driver line. Primers were specific to the *UAS-EGFP* transgene (UAS-EGFP), *CP-Gal4* transgene (CPT) and endogenous *CP* gene (CP). Actin primers were used as control (Actin). RNA samples were extracted from isolated digestive systems of female mosquitoes at different time points: 72-h-old mosquitoes prior to blood feeding (PV), 6, 12, 24 and 48 hours PBM.

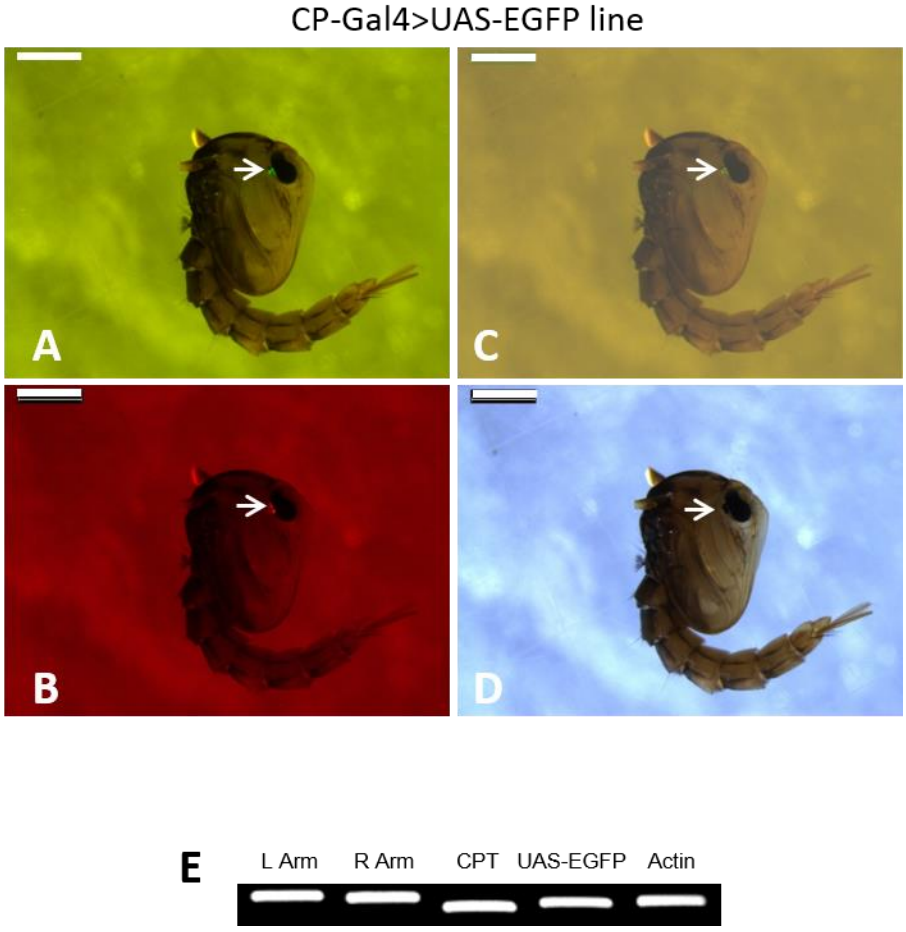

Figure 2.3. Establishment of the *Ae. aegypti* CP-Gal4>UAS-EGFP hybrid line. (A-D). Pupae of hybrid mosquitoes were screened for the presence of eye-specific expression of the selectable markers, EGFP and DsRed. (A) GFP-B filter; (B) DsRed filter; (C) merged; (D) white light. Scale bar: 1mm. Nikon SMZ800 fluorescence microscope with GFP-B and DsRed filters was used for screening. (E) Genomic PCR analysis for verifying the integration of the CP-Gal4 driver and UAS-EGFP responder constructs in the *Ae. aegypti* CP-Gal4>UAS-EGFP hybrid line. Specific primers used for the analysis: L Arm and R Arm – left and right arms of the piggyBac vector; CPT - *CP-Gal4* transgene; and UAS-EGFP - *UAS-EGFP* transgene. Actin probe was used as the loading control.

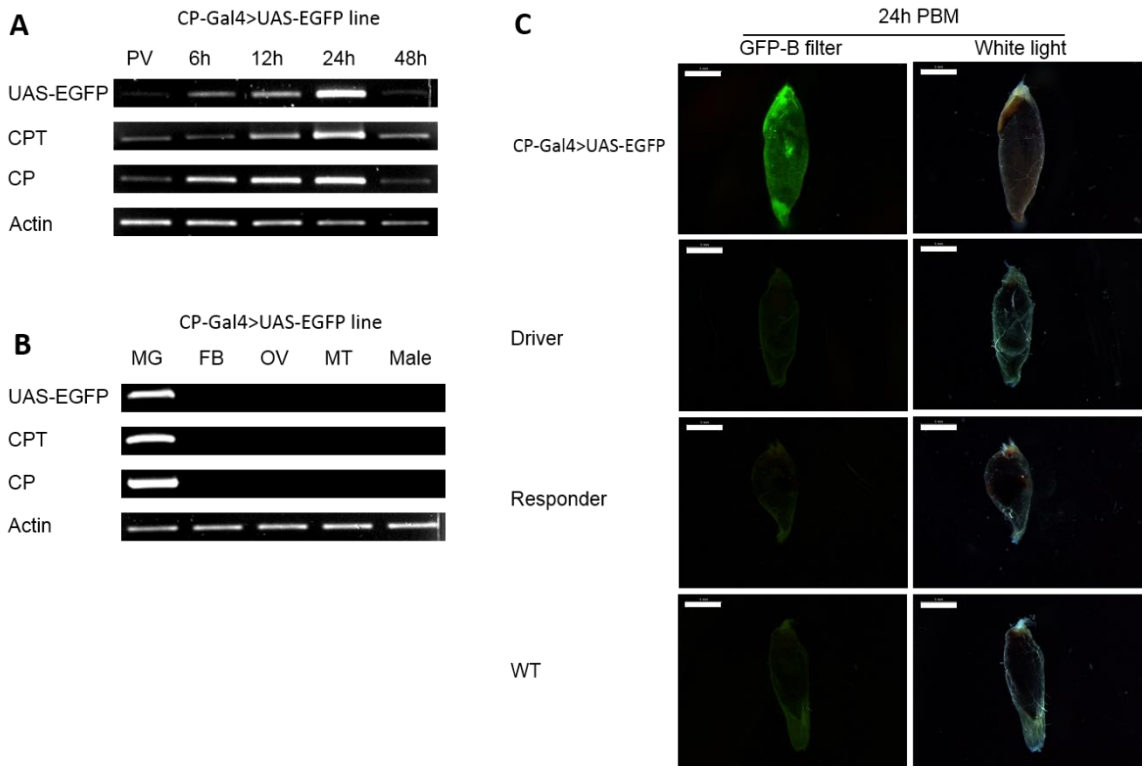

Figure 2.4. Expression of the *CP-Gal4>UAS-EGFP* transgene in the *Ae. aegypti* hybrid line. (A) The time course of transcript abundance in midguts of hybrid transgenic female mosquito line using gene-specific primers. RNA samples were extracted from different time points: PV, 6, 12, 24 and 48 h PBM. (B) Tissue- and sex-specific expression of the *CP-Gal4>UAS-EGFP* transgene in the CP-Gal4>UAS-EGFP hybrid line mosquitoes. RNA samples were extracted from midgut (MG), fat bodies (FB), ovaries (OV), Malpighian tubules (MT) of females at 24 h PBM and adult hybrid males (M). Analyses in (A) and (B) were performed by means of RT-PCR using UAS-EGFP, CPT and CP probes. Actin was used as a loading control in (A) and (B). (C) Representative image of EGFP expression in female midguts from the CP-Gal4>UAS-EGFP line, CP-Gal4 driver line M16-1 (driver), UAS-EGFP responder line (responder), and wild type (WT). Midguts were dissected at 24 h PBM. Images were obtained using Leica M165FC fluorescent stereomicroscope using GFP-B filter or transparent filter (White light) and LAS V4.0 software. Scale bar: 1mm.

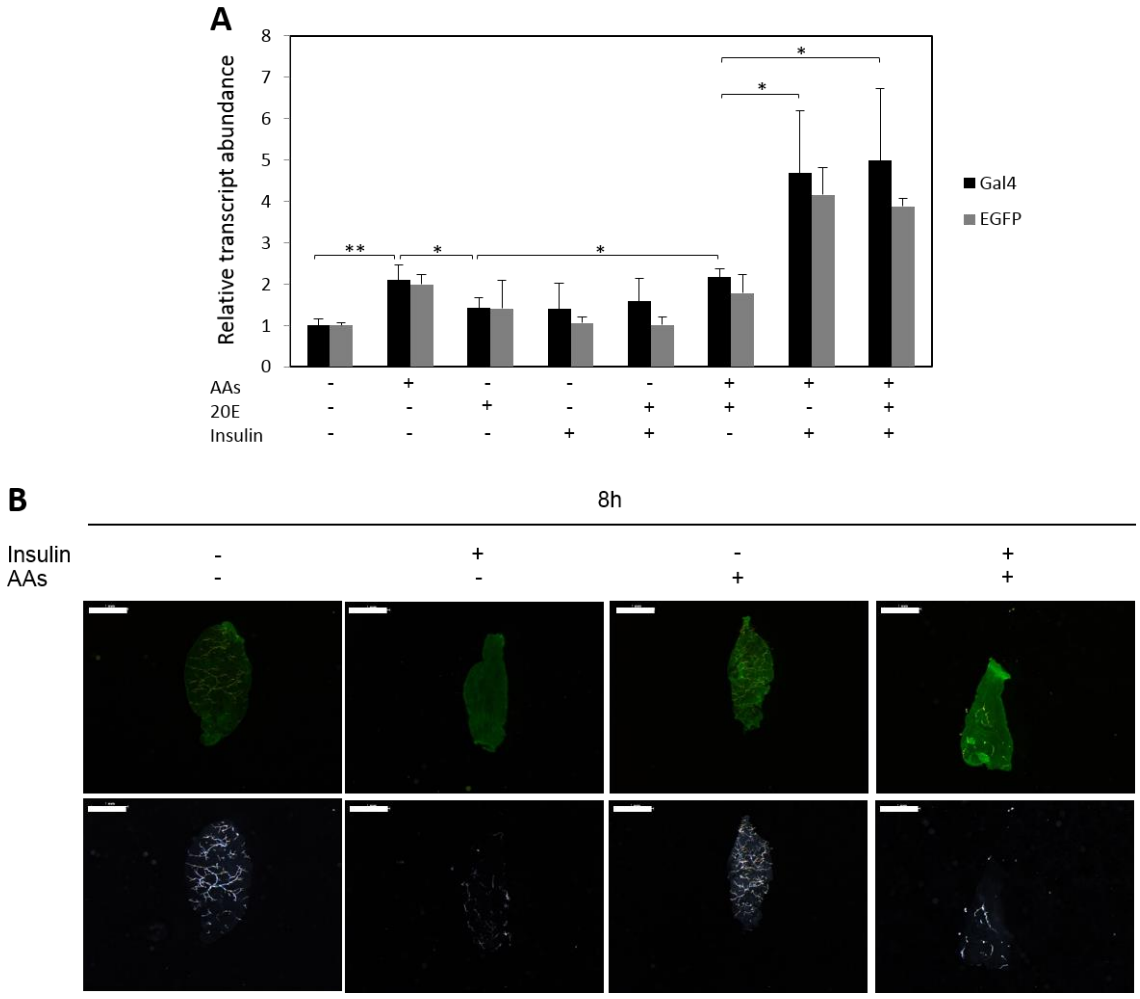

Figure 2.5. Effects of AAs, insulin and 20E on expression of the *CP-Gal4>UAS-EGFP* transgene. (A) Midguts were isolated from non-blood fed CP-Gal4>UAS-EGFP female mosquitoes and incubated under different indicated conditions *in vitro* for 8 h. Levels of the *CP-Gal4>UAS-EGFP* transgene transcript were monitored using Gal4 and EGFP primers (Table 2.S3) by means of qRT-PCR. Control transcript level was set at 1, while transcript levels for other treatments were expressed relative to the control. Values arethe means of three replicates (± SEM). The experiment was repeated three times. * Indicates statistical significance <0.05. ** Indicates statistical significance <0.01. (B) Detection of EGFP protein in midguts isolated from non-blood fed CP-Gal4>UAS-EGFP female mosquitoes by means of fluorescent immunocytochemistry. The midguts were cultured for 8h under indicated conditions. Immunocytochemistry was performed using the anti-EGFP antibody, followed by incubation with an anti-rabbit fluorescent secondary antibody. Images were obtained using Leica M165FC fluorescent stereomicroscope using GFP-B filter or transparent filter (White light) and LAS V4.0 software. Scale bar: 1mm.

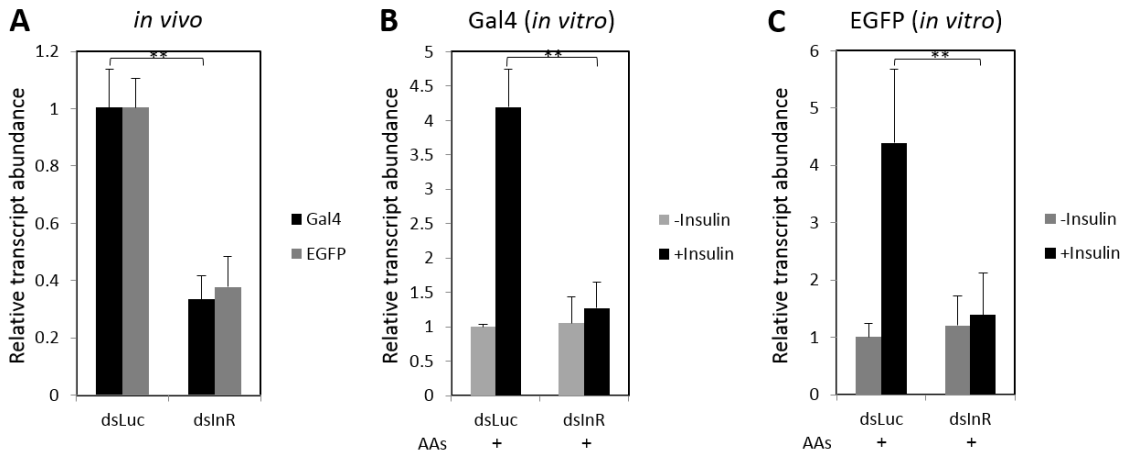

Figure 2.6. Insulin signaling pathway controls expression of the *CP-Gal4>UAS-EGFP* transgene. (A) RNAi of Insulin Receptor (dsInR)*in vivo*. RNAi for Luciferase (dsLuc) served as a control. The CP-Gal4>UAS-EGFP females were injected with dsRNAs at 1 day post-eclosion, blood fed and examined at 24 h PBM. Transcript abundance was examined using Gal4 and EGFP primers by means of qRT-PCR. Control dsLuc transcript level was standardized to 1, while transcript levels for other RNAi treatments were expressed relative to the control. (B-C). Activation of the *CP-Gal4>UAS-EGFP* transgene expression by insulin *in vitro* was prevented by dsInR. The CP-Gal4>UAS-EGFP females were injected with dsRNAs at 1-day post-eclosion, and an *in vitro* assay was performed as in Figure 5. Midguts were isolated from non-blood fed CP-Gal4>UAS-EGFP females and incubated inthe AA-containing culture medium. Expression of the *CP-Gal4>UAS-EGFP* transgene was monitored by means of qRT-PCR using probes for Gal4 (B) and EGFP (C) (primer sequences in Table 2.S3).

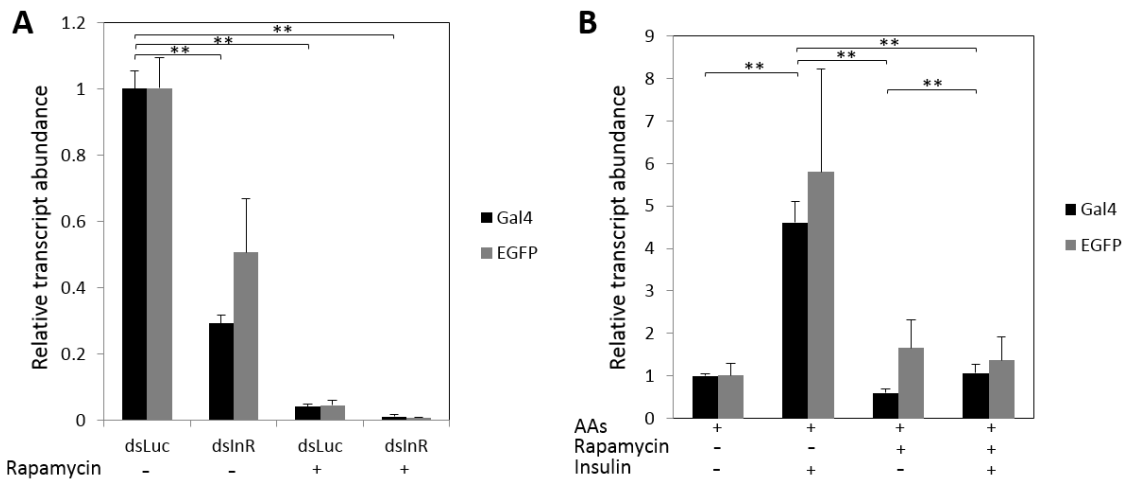

Figure 2.7. Effect of TOR inhibitor rapamycin on the expression of the *CP-Gal4>UAS-EGFP* transgene. (A). *In vitro* application of AAs and insulin activated *CP-Gal4>UAS-EGFP* expression, and rapamycin was capable of blocking this activation. The transcript level was set at 1 for the "no treatment" sample (control), and transcript levels for the other treatments were expressed relative to the control. (B). qRT-PCR analysis of *CP-Gal4>UAS-EGFP* transgene expression in midguts at 24 h PBM from CP-Gal4>UAS-EGFP hybrid females after different treatments. 20 pmol of rapamycin was injected in each experiment. Transcript levels were standardized to 1 for the dsLuc sample (control), and transcript levels for the other treatment were expressed relative to the control.

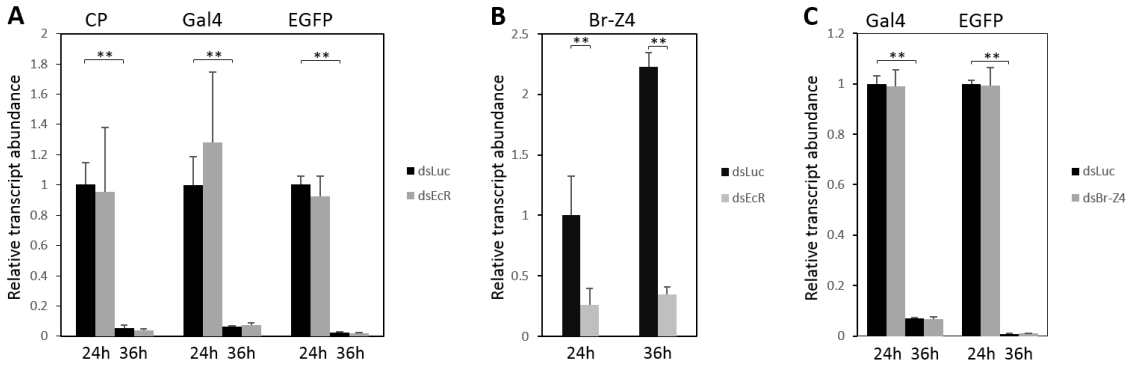

Figure 2.8. RNAi of the *EcR* (dsEcR) and *Br-Z4* (dsBr-Z4) gene in the CP-Gal4>UAS-EGFP female mosquitoes. (A-C). The procedure was performed as in Figure 6A. RNAi for Luciferase (dsLuc) served as a control. The effect of the *EcR* and *Br-Z4* knockdown on the *CP-Gal4>UAS-EGFP* transgene expression was monitored by means of qRT-PCR using Gal4 and EGFP primers. The transcript levels of the endogenous *CP* gene (A) and *Br-Z4* gene (B) after dsEcR treatment were also examined.Times at which examinations were done are marked by 24h and 36h PMB.

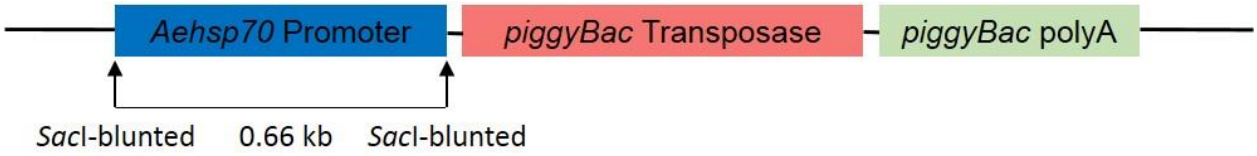

Figure 2.S1. A schematic representation of the pAehsp-pBac helper plasmid. Schematic representation of the pAehsp-pBac helper construct. This helper consists of the 0.66-kb *Aehsp70* promoter upstream of the *piggyBac* transposase coding region and polyadenylation.

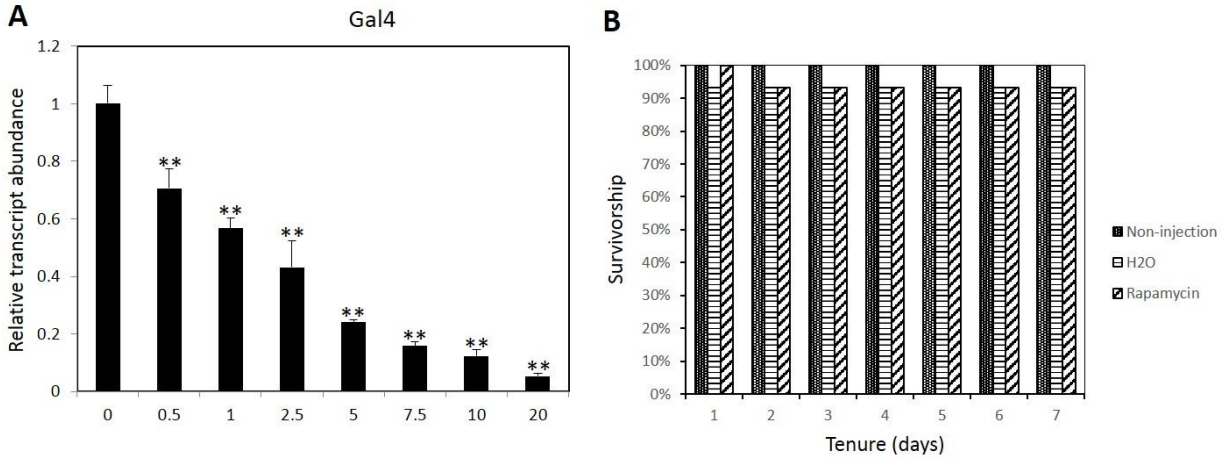

Figure 2.S2. Effect of rapamycin on transgene expression and survival in the hybrid female mosquitoes. (A) A dose–response effect of the TOR inhibitor rapamycin on expression of the *CP-Gal4>UAS-EGFP* transgene. Three-day-old hybrid female mosquitoes were injected with rapamycin (0–20 pmol) and blood fed 1 day later. The transcript level of the *CP-Gal4>UAS-EGFP* transgene was measured in midguts 24 h PBM by means of qRT-PCR analysis using the Gal4 primer. The transcript level of control mosquitoes injected with dH_2O was set at 1, while transcript levels for all other treatments were expressed relative to the control. Times designate injection amount (pmol). (B) Survivorship of female mosquitoes injected with 20 pmol of rapamycin. Non-injected mosquitoes served as a control. For each experimental set, the initial population size is 15 CP-Gal4>UAS-EGFP female mosquitoes. No difference was observed in survival between mosquitoes injected with H_2O and those treated with rapamycin. Times indicate days after injection.

No. of embryos injected	No. of G_0 survived to adulthood	No. oftransgenic lines	Transformation efficiency
1084	90	2	2/90=2.2%

Table 2.S1. Transformation data from *Ae.aegypti* lines using *piggyBac* transposable elements as insertion vectors.

Line	Location	Sequence
M16-1	39 kb from 5' end of AAEL010518	AATAACGTTATAAAAAAAAAC**TTAA-***piggyBac***-TTAA**AACTAAAGGCTCAACC
F4-4	8 kb from 5' end of AAEL004671	TGCAGTAACAGCTCTCTA**TTAA-***piggyBac***-TTAA**ACTTTATGTGTATCAGATAC

Table 2.S2. Genomic integration sites in *Ae.aegypti* transgenic lines M16-1 and F4-4.

Gene (PCR and RT-PCR)	Sequence 5'-3'
Actin	
Forward	AAGGCCAACCGTGAGAAGATGACT
Reverse	GCTCGTTGCCAATGGTGATGAC
CP	
Forward	GGGATTTGCCTATGTTTCGGAGT
Reverse	GCACCTCTGGCACGCTTCGA
CPT	
Forward (CPT-F)	TGGATCCGCGAGCTTGAAG
Reverse (CPT-R)	CGTTGCTACTGTTAGTGAAAGTGAA
UAS-EGFP	
Forward (UAS)	CAAGAAGAGAACTCTGAATAGGG
Reverse (158)	TGAAGTCGATGCCCTTCAGCT
piggyBac(Left Arm)	
Forward (L1)	TGTCAATGCGGTAAGTGTCA
Reverse (L2)	CCTCTGTGGCAAGGTCAAGA
piggyBac(Right Arm)	
Forward (R1)	TGATGACCTGCAGTAGGAAGACG
Reverse (R2)	AGAAACAACTTTGGCACATATCA
Inverse PCR (Left Arm)	
574F	TCTTGACCTTGCCACAGAGG
157R	TGACACTTACCGCATTGACA
Inverse PCR (Right Arm)	
2388F	GTCAGTCCAGAAACAACTTTGGC
2123R	CCTCGATATACAGACCGATAAAAACACATG
Aahsp70	
Forward	CTCGAGTCCAGTCTTTTTGAAGTCGCGAA
Reverse	AAGCTTCTTTAATTAGTGTTGTTTTGACGAGA

Gene (qRT-PCR)	Sequence 5'-3'
Actin	
Forward	GACTACCTGATGAAGATCCTGAC
Reverse	GCACAGCTTCTCCTTAATGTCAC
CP	
Forward	CTGGAGCAGTCGAGTGATAGTTTG
Reverse	CAATCTGACACGGACACCTTCG
Gal4	
Forward	TTCATCTTTCAGGAGGCTTGCT
Reverse	GAACTATAAATGGCACCTGATTGC
EGFP	
Forward	CAGGGTGGTCACGAGGGTGG
Reverse	GTGCCCATCCTGGTCGAGCT
InR	
Forward	GATAAACTGCGGGACATCGTG
Reverse	TGTCGGTGACGTCGATGGTA
EcR	
Forward	AAGCGAGGTTATGATGTTGCG
Reverse	CAGCAGGTCCTCTATCGTGTCC

Br-Z4	
Forward	TTCACCACAAGGTATGAGCACAG
Reverse	GCAGTCAGCGGAAGCGGT

Gene (dsRNA)	Sequence 5'-3'
dsLuc	
Forward	TAATACGACTCACTATAGGGCTCTGCCTCATAGAACTGCCTG
Reverse	TAATACGACTCACTATAGGGAACCTTCGCTTCAAAAAATGGA
dsInR	
Forward	TAATACGACTCACTATAGGGGAGATCACCGAGTACCTGCTGC
Reverse	TAATACGACTCACTATAGGGGCAACAGACCCCGGTCTTG
dsEcR	
Forward	TAATACGACTCACTATAGGGAAGCGAGGTTATGATGTTGCGAATG
Reverse	TAATACGACTCACTATAGGGTGAGGACGAGGACTGGGTGCC
dsBr-Z4	
Forward	TAATACGACTCACTATAGGGATCGGATTTAAACCACTCCAGCG
Reverse	TAATACGACTCACTATAGGGTGCGCAGTCTTTGTTGAACATCTAT

Table 2.S3. List of primers.

Chapter 3: Application of the Gal4/UAS system and microRNA Decoy technology: deciphering the target of microRNA-275 in *Aedes aegypti*

3.1 Abstract

The yellow fever mosquito *Aedes aegypti* transmits a number of devastating human diseases such as dengue fever, yellow fever and, recently, chikungunya fever. Female mosquitoes need to take vertebrate blood for their egg development, and repeated cycles of blood feeding are tightly linked with pathogen transmission. According to recent research, a number of endogenous microRNAs (miRNAs) are blood induced (Bryant et al, 2010). Although, it has been shown that microRNAs are involved in regulating diverse cellular functions, the functional roles of mosquito microRNAs remains unexplored. The miRNA aae-miR-275 (miR-275), in mosquitoes has been shown to be required for normal blood digestion. Systemic antagomir silencing of miR-275 resulted in severe phenotypes related to blood feeding and egg development (Bryant et al., 2010). However, the mechanism of miR-275 action remained unknown. Here we show, using a combination of the midgut-specific Gal4/UAS system and miRNA decoy technology, that endogenous level of miR-275 is highly reduced in the midgut of transgenic mosquitoes expressing miR-275 decoy after a blood meal. The lower level of miR-275 inhibited transcription of the mRNA encoding sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), resulting in disruption of normal blood digestion and egg production. Significantly, the level of miR-275 and SERCA expression in mosquito midgut correlates with blood digestion. These findings show the blood meal induced microRNA miR-275specifically functions in the midgut, where it stabilizes the transcript of the gene *SERCA* and assure blood digestive process to be accomplished normally.

3.2 Introduction

The yellow fever mosquito, *Aedes aegypti*, is distributed in tropical and subtropical areas worldwide and is the major vector of dengue fever, yellow fever and chikungunya viruses and some parasites. As an anautogenous mosquito, the female *Ae. aegypti* needs to gain nutrients from a blood meal to produce eggs, and pathogens are ingested as a result of feeding on an infected blood and infect the midgut transforming the female mosquito to be a machine of disease transmission. Therefore, interpreting the underlying mechanism for blood feeding and reproduction is crucial to developing new control strategies against the spread of mosquito-borne diseases. Recent studies have implicated a blood meal initiating global changes in the transcriptome; and shown that these changes are not limited to only protein-coding mRNAs, but also a large number of non-coding RNAs (ncRNAs), yielding a complex combination of transcripts that transact genetic information. ncRNAs appear to comprise a regulatory network that control gene expression at various levels of physiology and development. A large portion of these ncRNAs are microRNAs (miRNAs) (Akbari et al., 2013; Cech and Steitz, 2014).

miRNAs are small non-coding RNA molecules of ~21-nucleotide in length that play significant roles in post-transcriptional regulation of gene expression by forming hybrids with sequences located in the coding region or 3' untranslated regions (3' UTRs) of target messenger RNAs (mRNAs) (Hausser et al., 2013; Lucas and Raikhel, 2013). The general outcomes of miRNA-mRNA interaction are post-transcriptional repression by degradation of mRNAs and/or translational repression. However, new findings

indicate that miRNAs have the ability to stabilize mRNA or activate translation (Fabian et al., 2010; Hussain et al, 2011; Hussain et al., 2013). Recent studies have revealed that miRNAs play an important role in diverse biological functions such as blood digestion, reproduction, *Plasmodium* invasion, viral immunity and *Wolbachia* infection (Lucas et al., 2013). Bryant et al. performed the first functional analysis toward a mosquito miRNA in *Ae. aegypti*, showing that specific antagomir depletion of the conserved miRNA aae-miR-275 (miR-275) resulted in severe defects in intake and digestion of blood, fluid excretory function and egg development(Bryant et al., 2010). However, the precise roles of miR-275 in gonadotrophic cycle and gene targets contributing to these phenotypes have not been identified.

In *Ae. aegypti*, a number of miRNAs have shown to have sex-, tissue- and stage-specific expression. For example, in the Asian tiger mosquito *Aedes albopictus*, several miRNAs exhibit specifically increased expression at different developmental stages and sex, such as aal-miR-286b in embryos, aal-miR-2942 in larvae and aal-miR-1891 in adult females. Silencing of these three miRNAs by corresponding inhibitors resulted in a dramatic reduction in the hatching rate of embryos, the eclosion percentage of larvae and longevity and fecundity of adults, respectively (Puthiyakunnon et al., 2013). In another study, miR-989 displayed predominate expression in the ovaries in two mosquito species, *Ae. aegypti* and *Anopheles stephensi*. Furthermore, many miRNAs displayed up regulation in the female mosquito midgut and fat body in response to blood feeding (Li et al., 2009; Bryant et al, 2010). As various miRNA expression patterns are being uncovered, researchers encounter a rising challenge in discovering miRNA contributions

to numerous physiological, developmental and other biological processes. Although genomic and bioinformatics methods accelerate identification of new miRNAs, limitation of available technologies for *in vivo* study restrict exploration of miRNA functions.

In recent years, biologists developed multiple novel tools to study miRNA functions *in vivo*. For example, miRNA overexpression studies provided a body of evidence about miRNA functions in *Drosophila* (Bushati and Cohen, 2007). However, the significance of such gain-of-function experiments is generally limited because every miRNA have multiple target mRNAs (Lim et al., 2005). Mis-expression may result in neomorphic phenotypes because expressed miRNA may silence or enhance genes that normally escape regulation because of spatiotemporal or quantitative limits (Bushati and Cohen, 2007). Therefore, loss-of-function approach is necessary to validate gain-of-function results and/or investigate further on target miRNAs. The approaches that are currently used for loss-of-function analysis fall into two categories and they have their own caveats: those that compromise miRNA expression by manipulating the genome are time-consuming and flexibility-limited, those that silence mature miRNAs by chemically modified synthetic oligonucleotides (for example, antagomir) have a limitation for spatial analysis. A newly designed method, termed miRNA tough decoy (TuD), was used to express short transcripts consisting of a hairpin structure containing an internal bulge exposing multiple binding sites for specific miRNA-of-interest (Haraguchi et al., 2012). Meanwhile, Gal4/UAS system could provide robust spatiotemporal specific transgene expression (Elliott and Brand, 2008). Combination of these two technologies have

become a versatile tool that offer a promising advance in achieving spatial and temporal analysis to uncover miRNA functions in living organisms.

In the present study, we generated transgenic miRNA decoy by placing six tandemly arranged TuD hairpins downstream of upstream activation sequences (UASs). We have demonstrated that combining this construct with the midgut-specific CP-Gal4 driver achieves spatiotemporal suppression of miR-275 by the TuD miRNA inhibitor in mosquito. We further found that in the background of reduced miR-275 expression level, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) expression was down-regulated, while miR-275 mimic rescued normal SERCA expression in transgenic mosquitoes. Knock-down of SERCA and injection of SERCA specific inhibitor Thapsgargin exhibited a phenotype similar to that of the CP-Gal4>UAS-miR-275 Decoy hybrid transgenic mosquitoes. Luciferase assay confirmed that miR-275 stabilizes SERCA expression by binding to its 3'UTR. These results implied that miR-275 serves as a "protector" of SERCA in processes of blood digestion in midgut of mosquito *Ae. aegypti*.

3.3 Materials and Methods

3.3.1 Animals

The wild-type mosquito used was *Ae. aegypti* TF@K.Qnbj dedkkdq rsq`hm- Sgd v hkc-sxod `mc sq`mrfdmhb rsq`hmr v dqd l `hms`hmdc `s 16 ℃ with 80% relative humidity. Adults were reared with unlimited 10% (wt/vol) sucrose solution and water. There- or four-days after eclosion, the female mosquitoes were fed on the blood of White Leghorn chickens.

3.3.2 Plasmid Construction

The UAS-miR-275 Decoy construct AAEL-UAST_Decoy-linker-aae-mir-275-3P (t5) was a kind gift from Dr. Chun-Hong Chen (Institute of Molecular and Genomic Medicine, National Health Research Institutes, Taiwan). The AAEL-UAST_Decoy-linker-aae-mir-275-3P (t5) was used as a responder plasmid in transgenesis and it was produced based on the UAS-EGFP Responder (Kokoza and Raikhel, 2011), and the EGFP coding region was replaced with the miR-275 TuD cassette. A 1.3 kb fragment from the SERCA 3'UTR containing the predicted miR-275 binding site was cloned into psiCHECK-2 vector (Promega) downstream of a *Renilla* luciferase (*hRluc*) using *Pme*I and *Not*I restriction sites, resulting in a psiCHECK-2-SERCA construct. In addition, construct containing a mutated miR-275 binding site within the SERCA 3'UTR was also produced, yielding a psiCHECK-2-ΔSERCA construct. Empty psiCHECK-2 vector served as another control construct.

3.3.3 Germ-line Transformation of *Ae. aegypti*

The responder and helper plasmids were purified using the EndoFree Plasmid Maxi Kit. Responder (0.35mg/ml) and phsp-pBac helper (0.25mg/ml) plasmids were re-suspended in injection buffer (pH 6.8, containing 5mM KCl). The plasmid mixture was then injected into pre-blastoderm-stage eggs. The development of transgenic lines was performed as described previously (Kokoza and Raikhel, 2011). G1 progeny were screened by DsRed fluorescent marker in the eyes. The CP-Gal4>UAS-miR-275 Decoy

hybrid lines and were Vg-Gal4>UAS-miR-275 Decoy hybrid lines established as described previously (Kokoza and Raikhel, 2011). The hybrid mosquitoes exhibited EGFP and DsRed eye-specific selectable markers. In all screening experiments, a Nikon SMZ800 fluorescence microscope fitted with DsRed and GFP-B filter sets was used.

3.3.4 Molecular Analysis

DNeasy tissue kit (QIAGEN, Valencia, CA) was used to extract genomic DNA from adult mosquitoes. A total of 200 ng genomic DNA was used as template in PCR on the F1-1 and F17-1 responder lines using the Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). The PCR was performed at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, and a final extension at 68 °C for 7 min.

3.3.5 mRNA and miRNA Expression Analysis

miR-275 level was examined quantitatively by mean of quantitative reverse transcription PCR (qRT-PCR). QIAGEN miScript reverse transcription kit and miScript SYBR Green PCR kit were used to perform qRT-PCR. Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) from 10 midguts of blood-fed female mosquitoes at a serial of different time points. 1 μg of total RNA was treated by DNase I, and subsequently subjected to cDNA production using the miScript reverse transcription kit in a total 20-μl reaction mixture. Expression level of selected transcripts was determined by RT-PCR using Platinum PCR SuperMix. The amplification was performed under following conditions: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68

°C for 30 s, and a final extension for 7 min at 68 °C. Level of miR-275 and selected transcripts was checked by using miScript SYBR Green PCR kit (qRT-PCR) and qRT-PCR condition was as follows: 95 °C for 15 min, and 40 cycles of 94°C for 15 s, 60°C for 30 s and 70°C for 30 s, followed by the melting curve (65°C-95 °C). t-test was applied to compare sample means between different treatments. In these experiments, the ribosomal protein S7 (RPS7) gene was chosen as an internal control gene. Analysis is calculated as relative level to *RPS7* as described previously (Bryant et al., 2010).

3.3.6 miRNA Target Studies

Five programs, In-house, miRANDA, PITA, TargetScan and RNAhybrid were used to find potential targets of miR-275 in the *Ae. aegypti* genome.

MiR-275 mimic (5'-UCAGGUACCUGAAGUAGCGC-3') and control mimic (5'-UCACAACCUCCUAGAAAGAGUAGA-3') were synthesized by QIAGEN and used in transfection experiments at a concentration of 100 nM. The transfection was performed in Drosophila S2 cell line by Attractene reagent (QIAGEN). Luciferase activity was measured at 48h after transfection using three biological replicates, each with three technical replicates. SERCA Inhibitor Thapsgargin was purchased from Alomone labs and prepared as 100 mM stock solution in dimethylsulfoxide (DMSO). 0.5 µL of working solution at a concentration of 250 µM was injected into mosquitoes after blood meal.

3.3.7 Mosquito injection and RNA Interference

Injection of miR-275 mimic, control mimic and double strand RNA (dsRNA) was carried out in 1 day-old cold anesthetized female mosquitoes. Specific region of *SERCA* gene was amplified by PCR using gene-specific primers. The PCR product was used as template to synthesis double-strand RNA by MEGAscript kit (Ambion). Mosquitoes were injected 0.5 μL dsRNA at a concentration of 4 μg/μL and 0.5 μL mimic at a concentration of 200 μM. dsRNA from a region of *Luciferase* gene (dsLuc) was used as negative control. Mosquitoes were blood fed 3-4 days after injection.

3.4 Results

3.4.1 Development of CP-Gal4>UAS-miR-275 Decoy Mosquitoes

The CP-Gal4 driver line was utilized to provide midgut specific-, blood induced expression of Gal4 in this experiment (Fig. 3.1A). *piggyBac* transposable element was used to generate the UAS-miR-275 Decoy transgenic lines. In the UAS-miR-275 Decoy construct it contained the UAS that was linked to a miR-275 Tough Decoy sequence, followed by a SV40 polyadenylation signal. The transgenic cassette was assembled with *DsRed* marker gene driven by the eye-specific *3×P3* gene promoter and be adjacent to SV40 polyadenylation terminator (Fig. 3.1B).

The embryos of *Ae. aegypti* UGAL/Rockefeller strain (wild-type) were injected with a mixture of the UAS-miR-275 Decoy construct and helper plasmid. Around 1000 embryos that were injected with plasmids mixture, 208 G_0 mosquitoes survived to adulthood (127 males and 81 females). G_0 male was out-crossed individually with 5 wt

females, forming 127 male families (M1-M127). G_0 female was out-crossed with 1 wt male giving rise to 81 female families (F1-F81). DsRed marker was used to screen positive G_1 progeny (larvae and pupae) and two individuals (F1-1 from F1 female family and F17-1 from F17 female family) were selected based on the presence of strong DsRed expression in their eyes (Fig. 3.1C-E). The progeny of these two chosen individuals was established as homozygous.

CP-Gal4>UAS-miR-275 Decoy hybrid mosquitoes were produced by crossing CP-Gal4 driver line with both UAS-miR-275 Decoy responder lines. CP-Gal4>UAS-miR-275 Decoy hybrid mosquitoes exhibited the coincident presence of EGFP and DsRed eye-specific markers (Fig. 3.2A-D). The presence of *piggyBac* transposon left arm, right arm, CP-Gal4 driver sequence and DsRed-UAS responder sequence was confirmed by PCR analysis (Fig. 3.2E).

3.4.2 miR-275 Tough Decoy Down-regulates miR-275

To assess whether miR-275 Decoy construct worked efficiently in hybrid female mosquitoes, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to analyze miR-275 expression in the midgut. Comparing with control mosquitoes (wild-type, driver and responder lines), miR-275 expression levels was reduced to 30% in both CP-Gal4>UAS-miR-275 Decoy hybrid lines24 hours post blood-meal (PBM). *RPS7* was used as reference gene (Fig. 3.3). This data indicated that miR-275 expression was efficiently inhibited by miR-275 Tough Decoy.

CP-Gal4>UAS-miR-275 Decoy hybrid mosquito females with down-regulated miR-275 in the midgut presented dramatic defects in blood digestion and egg development. These phenotypes were characterized by undigested blood in the midgut, smaller follicle size, abnormal egg development and lower number of eggs. In both hybrid lines, blood remained partially undigested in the midgut (light red bolus) 24h PBM, but at the same developmental time, normal digestion (dark brown bolus) was observed in the wild-type mosquitoes, as well driver and responder lines (Fig.3.4A). In wild-type, driver and responder lines, the primary follicles averaged around 210μm in length 24h PBM with very small nurse cells at apex of the follicle. However, in both hybrids, the length of primary follicles dropped to around 170μm and nurse cells were substantially larger compared to controls (Fig. 3.4B). In addition, eggs showed smaller size and some of them displayed a lighter color (Fig. 3.4A). Another defect in the hybrid was that their number of eggs was reduced to around 30 per female, in comparison to controls being around 100 eggs per female (Fig. 3.4C). The similarity of phenotype in both hybrid lines indicated the defects caused by miR-275 depletion was solid and convincible.

3.4.3 miR-275 Regulates Expression of a Ca^{2+}-ATPase Gene

We next sought to find out the target gene(s) for miR-275. Five miRNA target prediction programs were used to identify miR-275 potential targets in *Ae. egypti*. Among hundred targets predicted by In-house, miRANDA, PITA, TargetScan and RNAhybrid search programs, one gene-sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-was

detected by all five of them. The *SERCA*-encoded mRNA contains a 3'UTR element that is partially complementary to miR-275. Mammalian orthologue of SERCA has been shown to be essential for muscle contraction and relaxation; also it is a major determinant of luminal environment which is critical for almost all local enzyme activities. Bryant et al. had been shown muscle weakening in the background of systemic antagomir depletion of miR-275 in female mosquitoes (Bryant et al., 2010). These results give us a clue that miR-275 regulates midgut muscle activities through SERCA.

We performed qRT-PCR to monitor miR-275 and *SERCA* expression levels at a serial time points after the blood meal uptake. In this experiment, in midgut of CP-Gal4>UAS-miR-275 Decoy hybrid mosquitoes there were significant decreases in the miR-275 level as compared to those wild-type, driver and responder controls, as well *SERCA* (Fig. 3.5). Applying miR-275 mimic in the hybrid mosquito restores *SERCA* transcript level and rescued the phenotype. Indeed, injection of miR-275 mimic elevated the *SERCA* mRNA level in both wild-type and hybrid midguts, and this mimic successfully overcame the defect brought by miR-275 Decoy, such as recovered blood digestion in the midgut and larger follicle size (Fig. 3.6). In support of these results, we observed a clear relationship between miR-275 and its target gene-*SERCA*.

It is not clear that interruption of the miR-275 and its targets in other tissues could also produce defects on blood digestion and egg development. We focused our attention on the fat body, which is an essential organ in hormone regulation and egg maturation (Arrese and Soulages, 2010). We generated Vg-Gal4>UAS-miR-275 Decoy hybrid lines, in which miR-275 level was specifically reduced in the fat body. In the fat body of Vg-

Gal4>UAS-miR-275 Decoy hybrid, decoy of miR-275 did reduced the level of miR-275 (Fig. 3.S1A). However, we did not observe any defects, such as blood digestion, follicle size reduction (Fig. 3.S1B).

To determine whether SERCA is a direct miR-275 target, the SERCA 3' UTRs, one that includes a predicted miR-275 binding site and another with the mutated binding sequence, were cloned into psiCHECK-2 vector and downstream from the *Renilla* luciferase gene, resulting in the constructs psiCHECK-2-SERCA and psiCHECK-2-ΔSERCA, respectively. The original psiCHECK-2 vector acted as another control. Cotransfection of *Drosophila* S2 cells with miR-275 mimic significantly increased luciferase activity in cells transfected with psiCHECK-2-SERCA (Fig. 3.7). No changes in luciferase activity were exhibited in cells transfected with the mutated miR-275 binding site (psiCHECK-2-ΔSERCA) or the negative control vector (psiCHECK-2) in response to miR-275 mimic (Fig. 3.7). This data demonstrates that miR-275 specifically targets the *SERCA* gene and induces its expression.

3.4.4 Inhibition of SERCA Disrupts Blood Digestion

Double-strand RNA (dsRNA) induced knockdown of *SERCA* resulted in an apparent lack of blood digestion, inhibition of egg development, as well as reduced egg number in comparison to controls (Fig. 3.8). Thapsigargin (TG), which is a highly specific and well-characterized inhibitor of SERCA, was used to validate the observed phenotypes in *SERCA* knocked-down mosquito. TG treatment disrupted normal blood

digestion greatly reduced the length of follicle and egg number. TG itself had no observed defect on mosquito survivorship (Fig. 3.9).

3.5 Discussion

Blood digestion is undoubtedly a complicated process involving many genes and signaling pathways. To date, understanding of the principles underpinning this dramatic event has focused on genes, which are related to hormone regulation, nutritional signals and mechanisms of digestive enzyme synthesis. Bryant et al. were first to report that miR-275 had a close relation with blood digestion, fluid excretion and egg development (Bryant et al., 2010). They observed that depletion of miR-275 by specific antagomir resulted in inhibition of ovary maturation and defect in muscle function in the alimentary canal that caused undigested blood to enter into the anterior midgut and crop. However, the detailed mechanism(s) surrounding any role that miR-275 plays and the miRNA targets contributing to the phenotype have not been determined. In this work, we unveiled the direct target of miR-275 and defined the involvement of this miRNA in blood digestion by utilizing a combination of the blood-induced, midgut-specific Gal4 driver and Tough Decoy responder.

In the current study, two Tough Decoy responder lines were established which contained UAS-miR-275 Decoy construct. The combination of CP-Gal4 driver and responders produced CP-Gal4>UAS-miR-275 Decoy hybrid mosquitoes. Compared with levels in control lines, miR-275 levels in hybrid midguts were significantly decreased after blood feeding. Silencing of miR-275 by midgut-specific expression of miR-275

decoy resulted in dramatic defects. These defects were consistent with the results reported by Bryant et al., who accomplished inactivation of miR-275 by antagomir injection after eclosion. Bryant et al. observed that in miR-275-ant background, anterior midgut was considerably wider and blood filled the crop, the frontal portion of digestive system used for storing nectar/sugur (Bryant et al., 2010). This phenotype suggests an impairment of muscle function in the anterior midgut. Indeed, our preliminary results displayed that in hybrid mosquito midgut, where reduced level of miR-275 was restricted in the posterior portion of digestive system, has muscle weakening. A potential reason for this difference may be related to the different range of depletion of miR-275. Antagomir which was injected into female mosquito caused a systemic decrease of miR-275 in the whole body, whereas transgenic expression of miR-275 decoy limited this reduction only to the midgut, but not foregut and crop.

Among the predicted target genes, SERCA was of a particular interest, because it is a Ca^{2+} ATPase that utilizes ATP as an energy source to transfer Ca^{2+} against the concentration gradients from the cytosol of the cell to the lumen of the sarcoplasmic/endoplasmic reticulum (SR/ER) (Hovnanian, 2007). SERCA plays a key role by restoring high Ca^{2+} concentration inside SR/ER after muscle contractions (Hovnanian, 2007). SERCA also found to be responsible for remaining high Ca^{2+} concentrations in the lumen of the ER, which is necessary for correct protein synthesis, folding, processing or trafficking through the ER/Golgi compartment (Periz et al., 1999). Our data indicated that aberrant miR-275 level in midgut after blood feeding resulted in a statistically significant decrease of *SERCA* expression, which suggests impaired restoring

of Ca^{2+} in muscle and this data was consistent with the phenotype of abnormal muscle function. *SERCA* as a direct target of miR-275 was supported further by luciferase assay and the demonstration that injection of specific miRNA mimic of miR-275 in hybrid mosquitoes restored transcript level of *SERCA* and rescued the phenotype.

It has been established that miRNAs inhibit protein synthesis either by negatively regulating the expression and subsequent degradation of mRNA targets or repressing translation. However, some recent studies unveiled that some miRNAs have the ability to up-regulate transcript level of particular target mRNA or activate mRNA translation (Vasudevan et al., 2007; Vasudevan et al., 2008; Orom et al., 2008; Henke et al., 2008; Niepmann, 2009). In mosquitoes, it was reported that aae-miR-375 expression in *Ae. aegypti* is only detected after the uptake of a blood meal and it up-regulates transcript level of the Toll immune pathway components *Cactus* (Zhang et al., 2013). Another study demonstrated that *Wolbachia* uses a host microRNA, aae-miR-2940, to enhance transcript levels and/or the stability of the mRNA of *m41 ftsh* in *Ae. aegypti* (Hussain et al., 2011).

It was reported previously that *SERCA* is indispensable for maintaining high Ca^{2+} concentration in the ER, which is essential for protein synthesis and correct transport in *Drosophila* and human (Roti et al., 2013; Periz et al., 1999). In the *SERCA* mutants, Notch cleavage and receptor trafficking to the cell membrane were impaired. And deficiency in *SERCA* also causes many transmembrane proteins to mislocalize in tissue suggests SERCA plays a general role on protein transport (Periz et al., 1999). TG inhibition of SERCA interferes with early maturation of Notch1 in the ER (Roti et al.,

2013). Given the fact that knockdown of *SERCA* or application of TG disrupted blood digestion and egg development, we next need to ascertain whether reduction of *SERCA* level or its activity in midgut resulted in changes in the Notch signaling or abnormal digestive enzyme synthesis and/or transport and finally interfere blood digestion processes.

In summary, this is the first report on application of Tough Decoy technology in non-drosophilid insects. Here, we generated transgenic miRNA decoys by placing miR-275 complementary oligonucleotides downstream of UASs. Our current results indicated that combining decoy with specific Gal4 driver delivers sufficient expression of the tough decoy to attain midgut-specific, blood induced miR-275 inhibition in female mosquitoes. We demonstrated that miR-275 is indispensable in blood digestion in midgut, by stabilizing a key target, Ca^{2+} ATPase SERCA. These results provide an opportunity to devise novel strategies against disease vectors.

3.6 References

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3.7 Figures and Tables

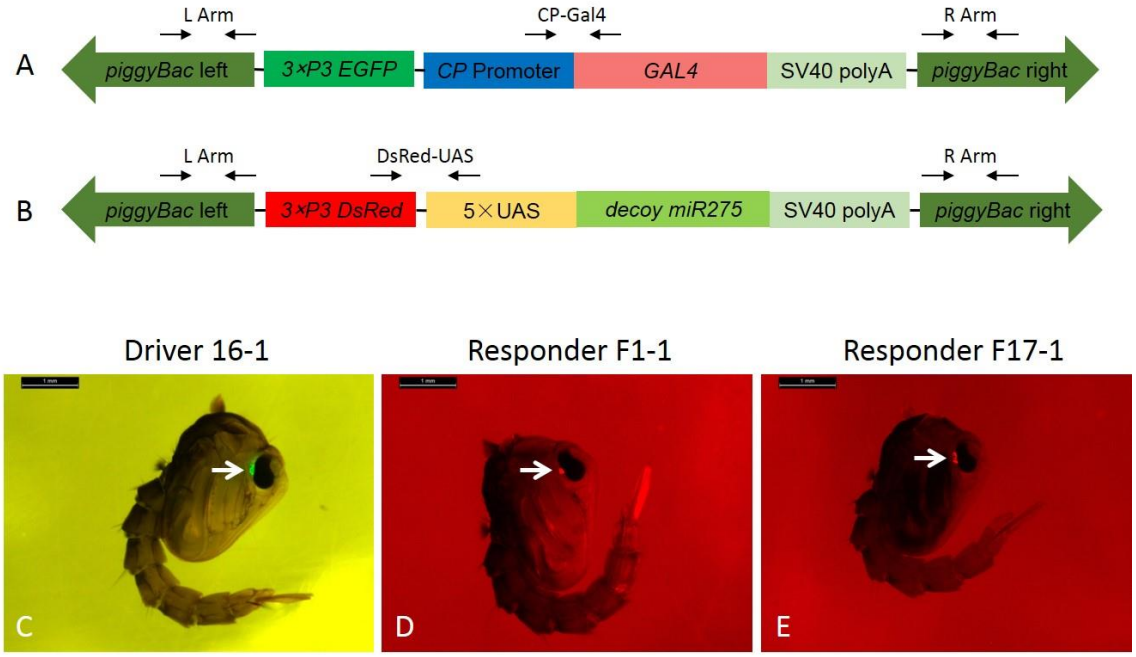


Figure 3.1. Stable incorporation of UAS-miR-275 Decoy construct in the *Ae. aegypti* genomes. (A) Schematic representation of the driver CP-Gal4 cassette. (B) Schematic representation of the responder UAS-miR-275 Decoy cassette. (C,D,E) Eye-specific expression of the selectable markers EGFP and DsRed in transgenic mosquito lines.

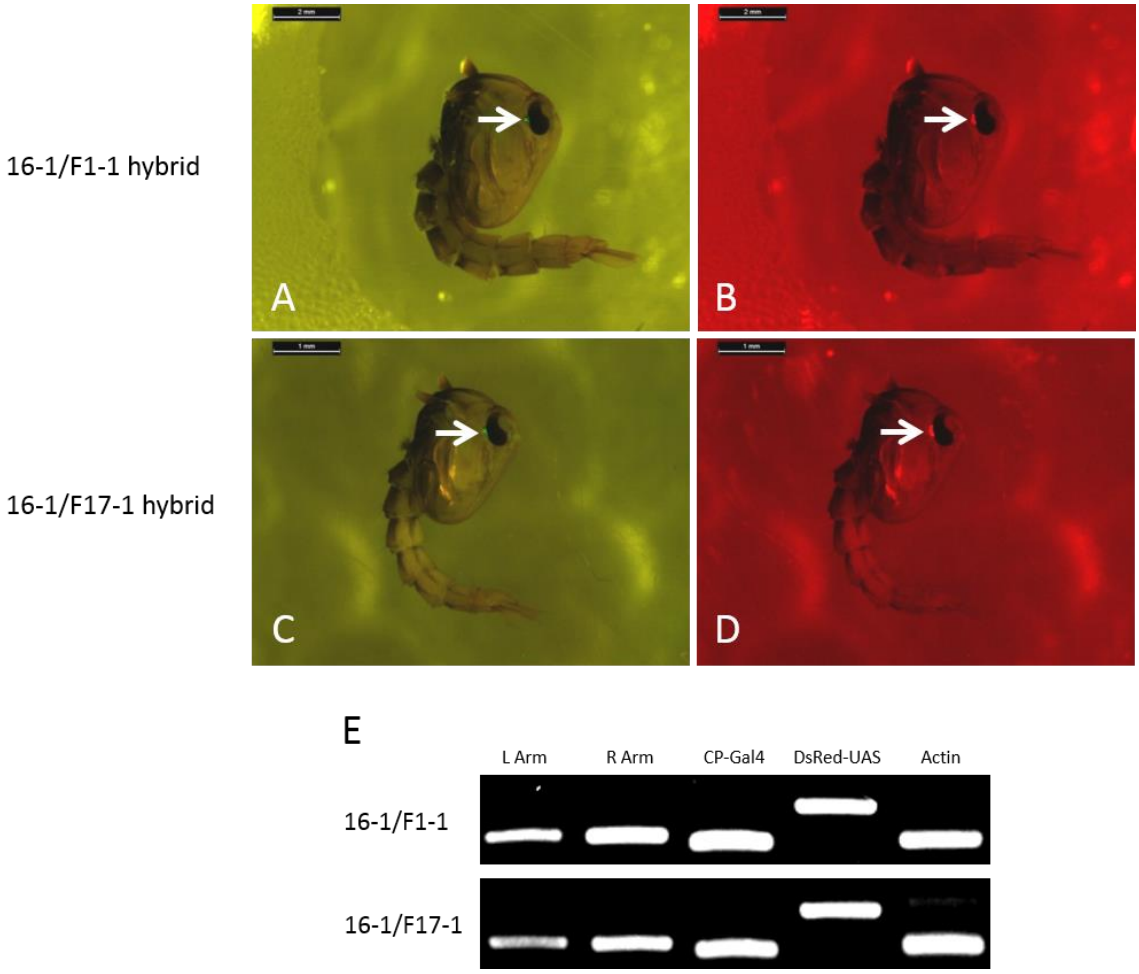


Figure 3.2. Confirmation of the hybrid lines. (A-D) Eye-specific expression of the selectable markers EGFP and DsRed in hybrid transgenic mosquitoes. (E) Stable incorporation and the integrity of UAS-miR-275 Decoy construct in the hybrid transgenic mosquitoes.

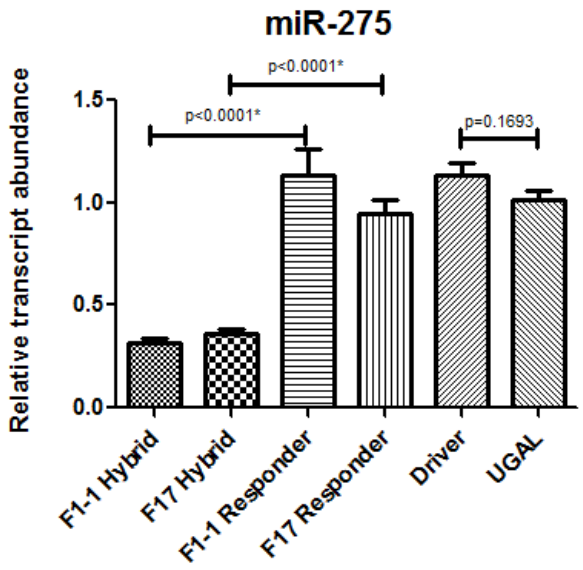
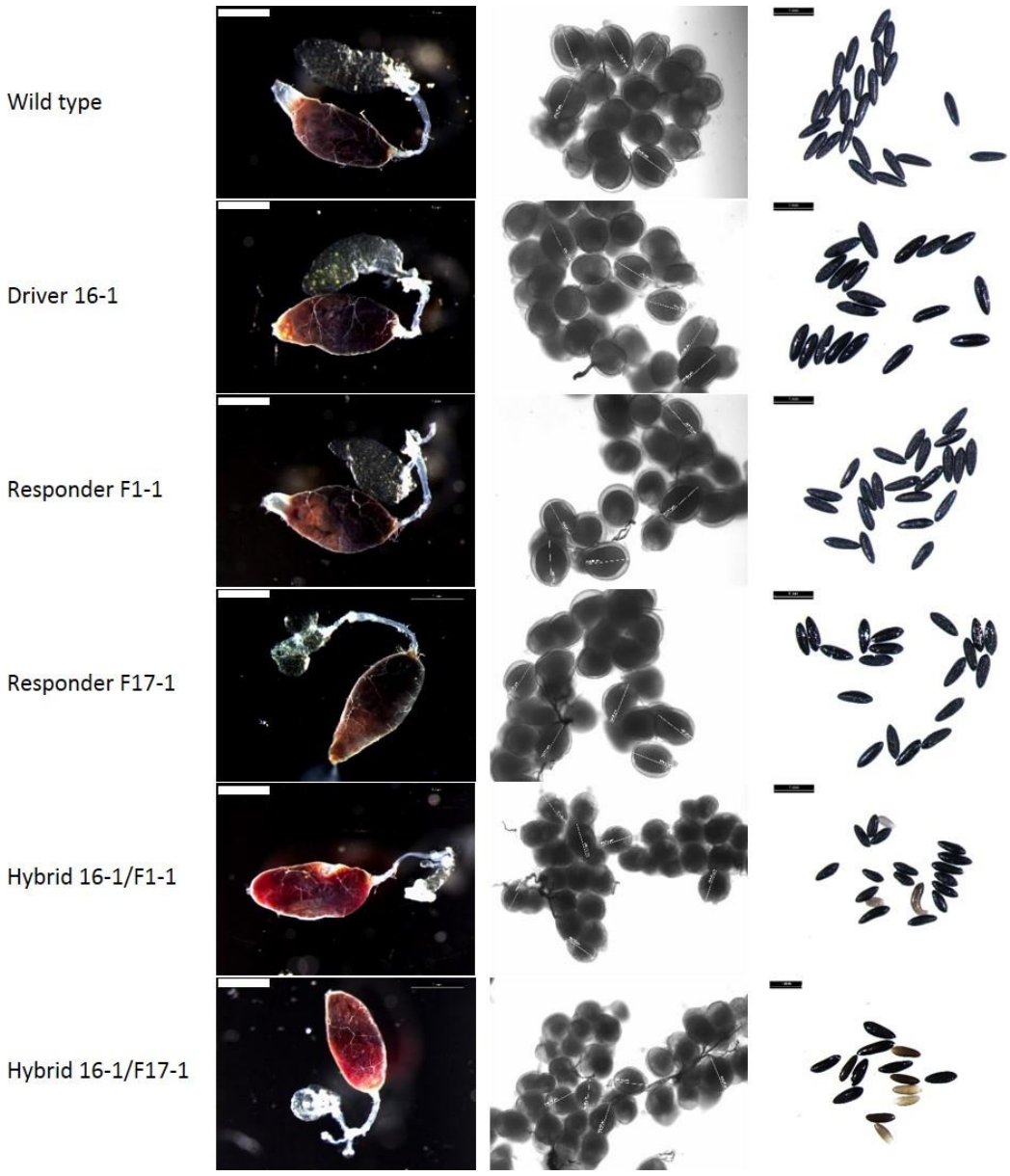


Figure 3.3. Expression level of miR-275 in midgut at 24h PBM. Midguts were isolated from blood fed CP-Gal4>UAS-miR-275 Decoy female mosquitoes 24h PBM. Expression of *RPS7* gene was used as control. Values are the means of three replicates (± SEM). The experiment was repeated three times.* Indicates statistical significance at $p < 0.05$.

A


Wild type
Driver 16-1
Responder F1-1
Responder F17-1
Hybrid 16-1/F1-1
Hybrid 16-1/F17-1

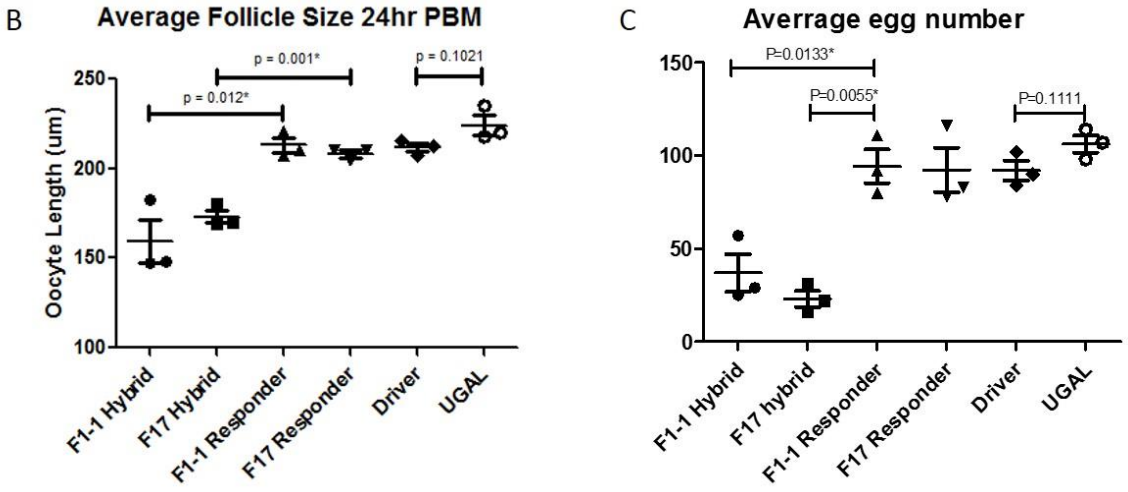


Figure 3.4. Phenotypes of two CP-Gal4>UAS-miR-275 Decoy hybrid lines, 16-1/F1-1 and 16-1/F17-1 lines. (A) Phenotypes of midgut, ovaries and eggs in wild-type (UGAL), CP-Gal4 driver, Responder F1-1, Responder F17, hybrid line 16-1/F1-1 and 16-1/F17-1. (B) Average follicle size in different lines at 24h PBM. (C) Average egg number in different lines. * Indicates statistical significance at $p < 0.05$.

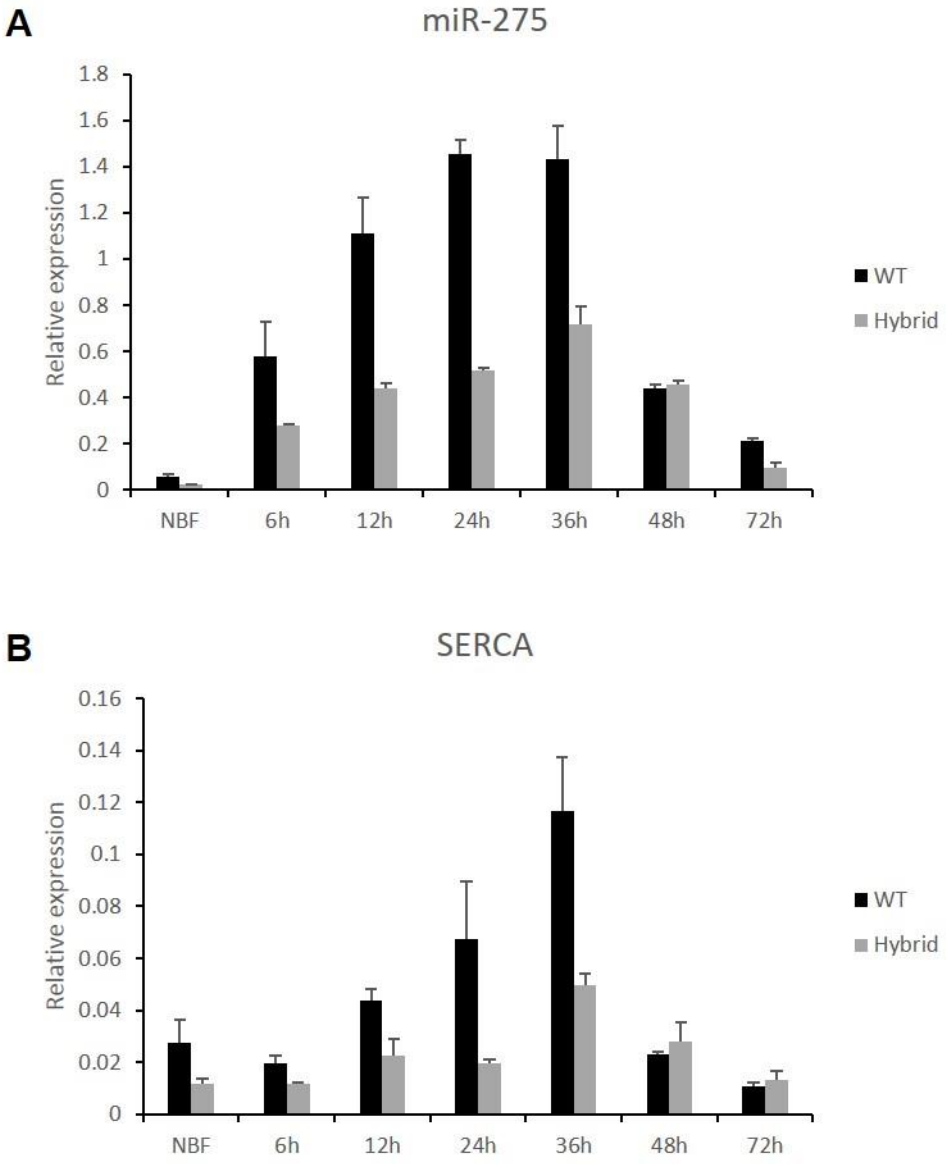
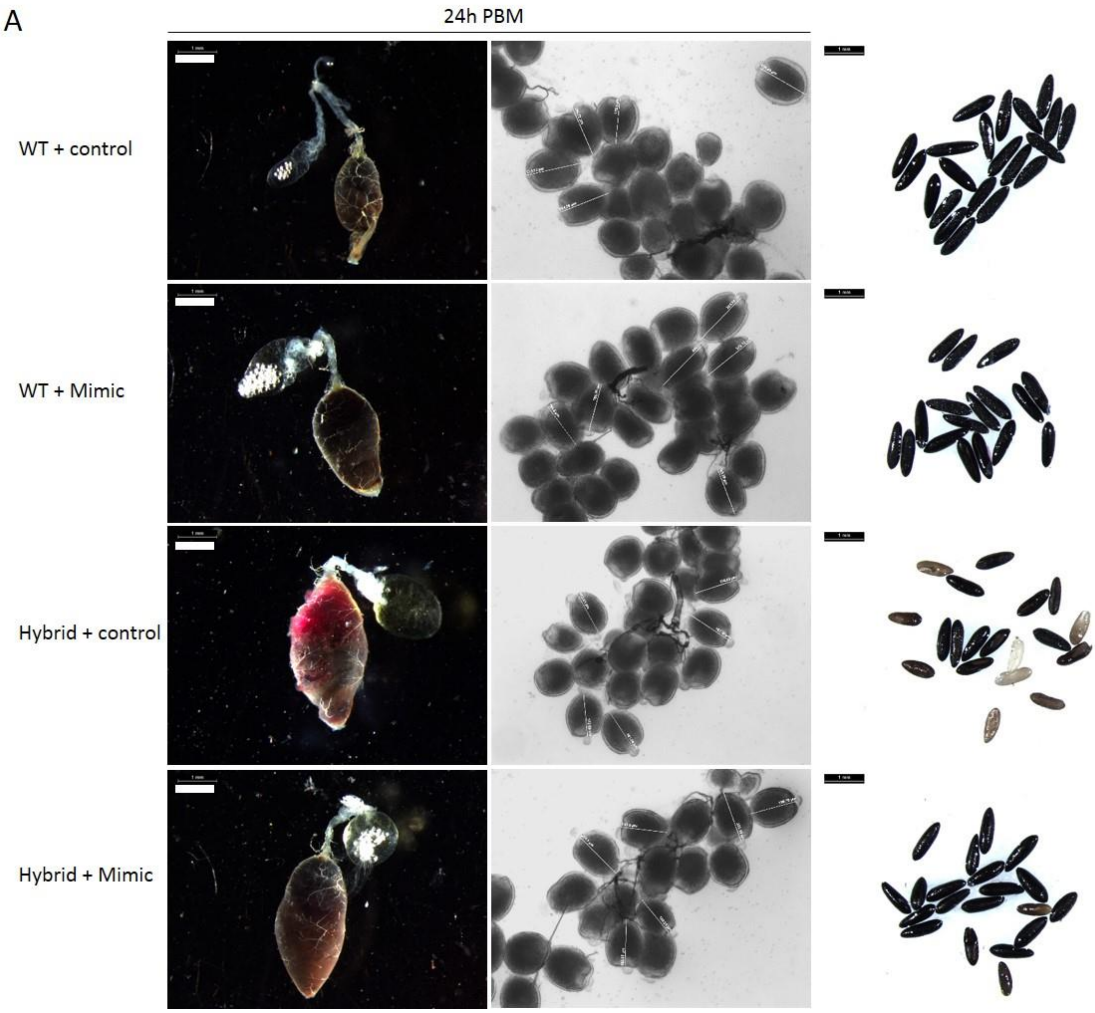


Figure 3.5. miR-275 and *SERCA* are down-regulated in hybrid midgut. (A) miR-275 expression levels in different time points in wild-type (WT) and hybrid midgut. (B) *SERCA* expression levels in different time points in wild-type (WT) and hybrid midgut. *RPS7* is used as reference gene.


A
24h PBM
WT + control
WT + Mimic
Hybrid + control
Hybrid + Mimic

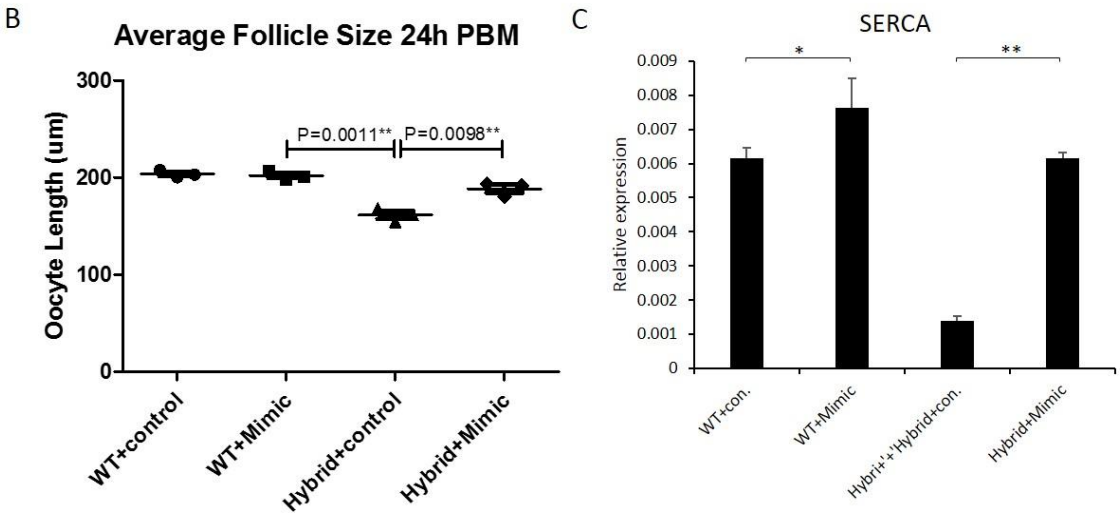


Figure 3.6. miR-275 mimic rescues the phenotypes in hybrid mosquitoes. (A) Phenotypes. (B) Average follicle size. (C) SERCA expression at 24h PBM. * Indicates statistical significance at $p<0.05$. ** Indicates statistical significance at $p<0.01$.

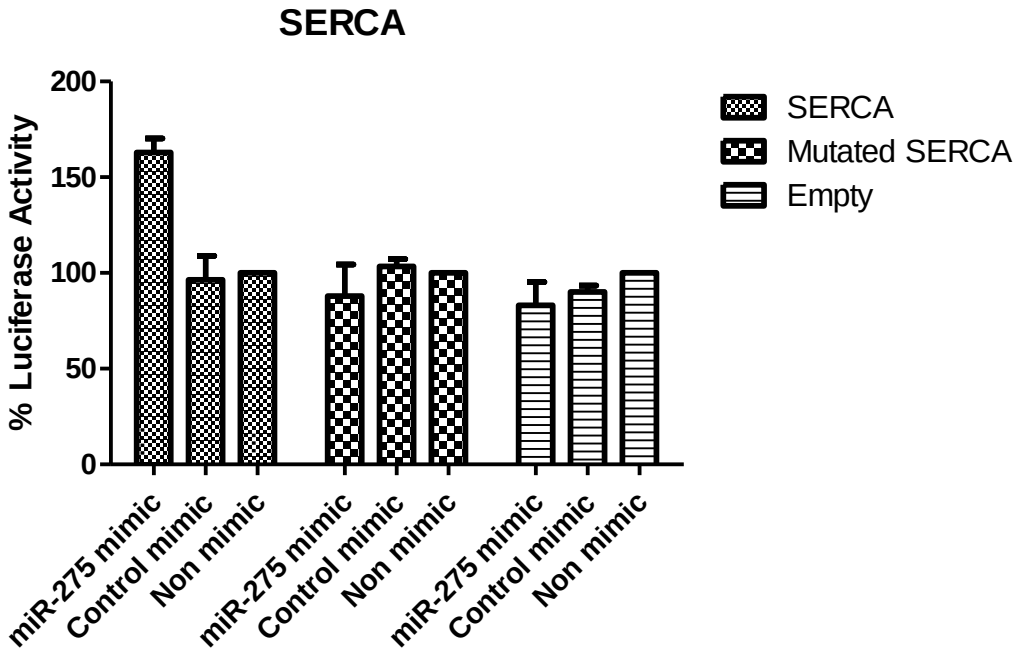


Figure 3.7. *SERCA* is a target gene of miR-275. Luciferase assay demonstrates that miR-275 binds 3'UTR of *SERCA* directly.

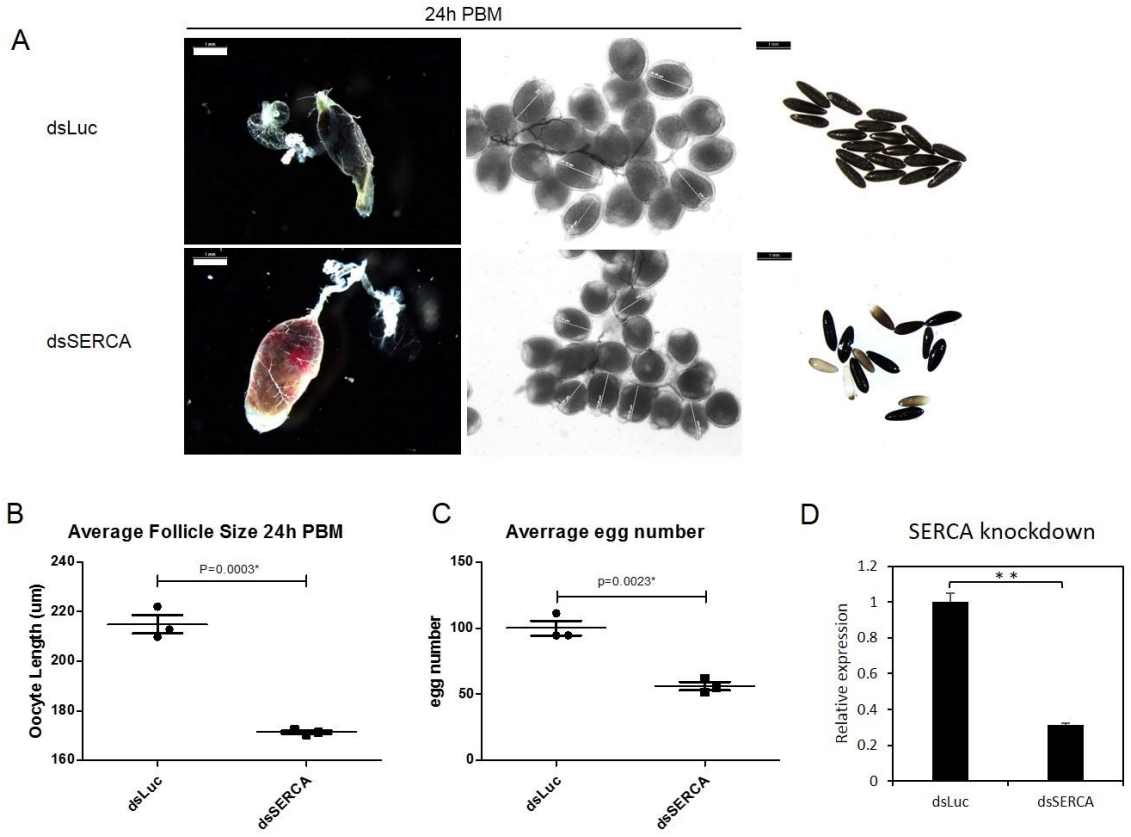


Figure 3.8. *SERCA* plays an important role on blood digestion and egg development. (A) Phenotypes of *SERCA* knockdown. (B) Average follicle size at 24h PBM after knockdown of *SERCA*. (C) Knockdown of *SERCA* reduces the average egg number. (D) *SERCA* knockdown efficiency at 24h PBM. ** Indicates statistical significance at $p<0.01$.

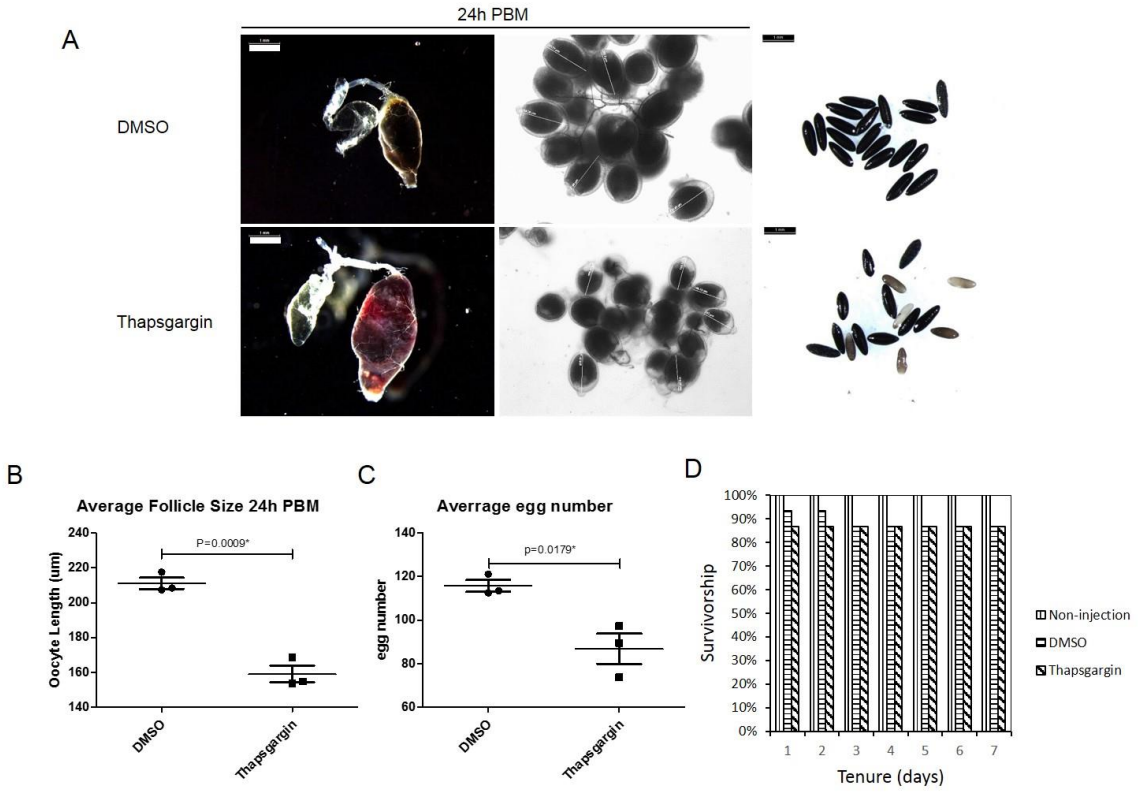


Figure 3.9. Application of SERCA-specific inhibitor Thapsgargin results in similar phenotypes as hybrids. (A) Phenotypes of mosquito by different treatments. (B) Average follicle size at 24h PBM. (C) Average egg number. (D) Survivorship after DMSO and Thapsgargin injection.

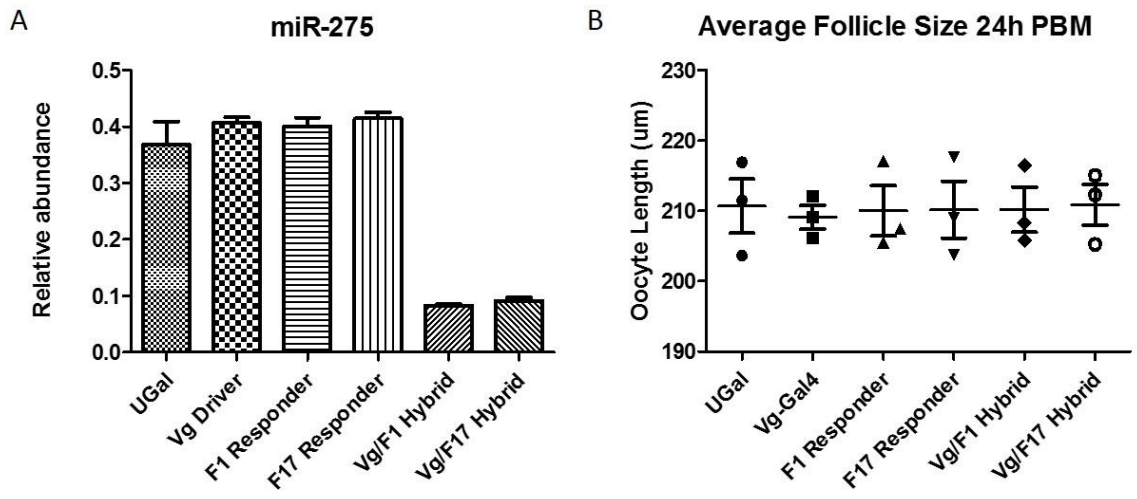


Figure 3.S1. Phenotypes of Vg-Gal4>UAS-miR-275 Decoy hybrid lines. (A) miR-275 level is reduced in Vg-Gal4>UAS-miR-275 Decoy hybrid lines compared to wild-type (UGAL), driver line and responder lines. (B) Follicle size in control lines and two hybrid lines, 24h PBM.

Chapter 4: Development of two migdut-specific Gal4 driver lines in *Aedes aegypti*

4.1 Abstract

Understanding of regulatory mechanisms involved in blood digestion and pathogen invasion is the foundation of developing novel mosquito control strategies. Refinement and development of the midgut-specific Gal4/UAS system provides a powerful tool to investigate the midgut biology. The goal of this study is to expand the midgut-specific Gal4/UAS system in *Aedes aegypti* by using promoter region of two genes whose expression is strongly induced in midgut before and after blood feeding. Here, we generated transgenic driver lines by placing *Ae. aegypti* early trypsin (AaET) and late trypsin (AaLT) regulatory elements upstream of transcription factor Gal4. We introduced the constructs contained transgenes into *Ae. aegypti* germ-line by *piggyBac* transposon.

4.2 Introduction

Anautogenous mosquitoes require blood for egg development and reproduction, and their cyclic blood feeding habits result in disease transmission. Diseases spread by mosquitoes such as malaria, Dengue fever, Yellow fever, West Nile Virus and Filariasis have become a major public health threat throughout the world. Previous control efforts led severe problems such as drug resistance in the *Plasmodium* parasites, rapid selection of insecticide-resistant strains of mosquitoes and environmental issues. The lack of effective vaccines has sharpened fears of numerous infectious and parasitic diseases. Although new drugs and therapeutic strategies under development show tremendous potential, there are no promising methods for the control mosquito borne-diseases till today (Beerntsen et al., 2000). As a consequence, we urgently need tools of molecular biology and genetics to pave avenues of research in mosquito biology and finally develop innovative control approaches.

The midgut is extremely important for mosquito not only because it is the organ of blood meal digestion and absorption but also it plays critical roles in immunity, metabolism, homeostasis, osmotic pressure and more. Midgut is crucial entry point for pathogens in infected blood such as malaria parasite *Plasmodium* and Dengue viruses. Understanding the regulatory mechanisms governing blood digestion and immunity greatly relies on tools such as germline transformation and midgut-specific Gal4/UAS system. Disrupting digestion processes by inhibition of key genes or enhancing immunity towards pathogens in midgut could be efficient strategies to control mosquito population

or disease transmission. To realize this goal, refinement and development of midgut-specific Gal4/UAS system is becoming indispensable and urgent.

After a blood meal, numerous endo- and exoproteolytic enzymes are synthesized, activated and released from midgut epithelial cells into the gut lumen where digestion occurs. Digestive products (amino acids) and other soluble substances such as lipids and carbohydrate are used for building up processes of yolk protein and egg development (Raikhel and Dhadialla, 1992). Different combinations of digestive enzymes are induced at early phase, which is from 1–3 h post blood meal (PBM) or the late phase beginning 8–36 h PBM (Felix et al., 1991). In *Aedes aegypti*, the principal enzyme during the early phase is early trypsin (AaET). *AaET* gene transcript is absent in newly emerged females, it reaches detectable levels 24 h post eclosion and accumulates to a maximum level in 2-3 days (Noriega et al., 1996a). After a blood meal, mRNA of AaET is immediately translated within 1 h PBM and becomes the most abundant midgut protein at 3 h PBM, then disappears by 6–8 h PBM (Noriega et al., 1996b). As a representative of late phase protease, late trypsin (AaLT) begins to appear 8–10 h PBM, and their abundance partly accounts for the endoproteolytic activity present in the midgut during blood-protein digestion (Barillas-Mury and Wells, 1993).

In this work, we plan to generate two transgenic lines, in which transgenes driven by regulatory regions of AaET or AaLT genes exhibit a temporal- and spatial-specific expression in *Ae. aegypti* mosquito.

4.3 Materials and Methods

4.3.1 Animal

Aedes aegypti mosquitoes (UGAL/Rockefeller strain and transgenic lines) were reared under following conditions: 27°C and 80% humidity, unlimited access to 10% sugar solution and water. Mosquitoes were fed on White Leghorn chickens to complete their gonotrophic cycle.

4.3.2 Plasmid Construction

A 1.4 kilobase (kb) fragment containing the AaLT 5' regulatory region was obtained by PCR with specific primers using genomic DNA as template, followed by digestion with *Bam*HI and *Bgl*II, then were inserted into the *Bgl*II site of pBac [3×P3-EGFP *afm*] transformation vector (Horn and Wimmer, 2000) to yield pBac[AaLT]. Plasmid pBac[AaLT-Gal4] was derived from pBac[AaLT] by inserting a *Bam*HI fragment containing chimeric Gal4 activator upstream of SV40 terminator fragment from pBac[3×P3-EGFP *afm*, *Vg-Gal4*] (Kokoza and Raikhel, 2011) into the *Bgl*II site (Fig. 4.1A).

A 2.1 kb promoter region of AaET was amplified by PCR and cloned into pCR4-TOPO vector (Invitrogen). This construct was digested with *Pme*I and *Spe*I, and linked with a *Pme*I-*Spe*I fragment containing Gal4 and SV40 polyadenylation signal from pBac[3×P3-EGFP *afm*, *Vg-Gal4*]. This AaET-Gal4-SV40 cassette will digest with *Asc*I and inserted into the *Asc*I site of pBac [3×P3-EGFP *afm*] transformation vector to yield pBac[AaET-Gal4] (Fig. 4.1B).

4.3.3 Germ-line Transformation of *Ae. aegypti*

Ae. aegypti embryos were injected with a mixture containing 0.35mg/ml of the transformation vector (pBac[AaET-Gal4] or pBac[AaLT-Gal4]) and 0.25mg/ml of the phsp-pBac helper plasmid, as previously described (Kokoza and Raikhel, 2011). G_1 progeny were analyzed on Nikon SMZ800 fluorescence microscope fitted with GFP filter to detect eye-specific EGFP expression.

4.4 Result

4.4.1 *Ae. aegypti* Transformation

The *AaET-Gal4-SV40* transgene consists 2.1 kb of *AaET* putative regulatory sequences including the 5' untranslated region (UTR) and the protein coding sequence of transcription activator Gal4, followed by the SV40 terminator fragment. *AaLT-Gal4-SV40* transgene shares a same structure with *AaET-Gal4-SV40,* but Gal4 gene was under the control of a 1.4 kb promoter region from *AaLT*. *AaLT-Gal4-SV40* transgene was cloned, together with a *3×P3-EGFP* marker cassette, and inserted into the arms of *piggyBac* transposable element, to generated pBac[AaLT-Gal4] vector and used in the germ-line transformation (Fig. 4.1A-B).

In the unfinished transformation experiments with pBac[AaLT-Gal4], of around 1000 pre-blastoderm embryos injected, 270 surviving larvae were reared to adults (G_0) and separated by sex with 120 males and 150 females. 150 females were separated into 6 groups, in each group, females were mated with equal number of wild-type male giving

rise to 6 different female families. In the next generation, G_1 progeny (larvae and pupae) were screened for the expression of eye-specific EGFP marker. Based on this criterion, 2 founder mosquitoes were selected from 2 distinct families so far.

4.5 Discussion

Mosquito midgut is the place where blood digestion occurs. But the midgut epithelium is not ready for blood immediately after eclosion, it needs 2−3 days to carry out post-emergence development including the formation of microvilli, the aggregation of rough endoplasmic reticulum to form a "whorls" structure and the elaboration of the basal labyrinth (Hecker et al. 1971). During the previtellogenic phase, with juvenile hormone (JH) titers increase, the "whorls" structure formation and which is a sign of increase in protein synthesis. Blood feeding triggers a rapid decrease in hemolymph JH titers meanwhile the "whorls" disappear (Rossignol et al. 1982). JH also activates transcription of AaET and a chymotrypsin-like serine protease JHA15 expression in the newly emerged female adults (Noriega et al. 1997; Bian et al. 2008). Interrupting genes which involve JH actions in previtellogenic stage in midgut could prevent midgut development and by this way to hamper their ability of blood digestion. In this work, we are expecting to generate the previtellogenic-, midgut and female-specific Gal4 driver which can facilitate us to identify key regulator in post-emergence development in midgut and dissect of JH signaling, and the blood-meal-induced, midgut and female-specific Gal4 driver will benefit us to investigate crucial genes which are requisite for blood digestion.

Midgut is a major portal by which pathogens invade mosquito body. When a mosquito ingests a blood meal contained infectious agents, pathogens such as *Plasmodium* parasite and Dengue virus cross the midgut epithelium, replicate, then liberate into the haemocoel. The development of the pathogens is completed then they disseminate to the salivary glands and the mosquito is prepared to disease transmission (Ghosh et al., 2000; Clyde K et al. 2006). To date, we have only a primary understanding of pathogen interactions with mosquito and the innate immune responses. Activation of endogenous innate immune genes in transgenic mosquitoes may impose a fitness cost (Hurd et al., 2005). Modification of essential genes related to anti-pathogen can have potentially harmful effects on fitness. To address these disadvantages, by employing the midgut-specific Gal4/UAS system, we will have effective tool to investigate function of specific genes that allow the highlevels of inducible expression of anti-pathogen factors in midgut prior and/or after the blood feeding.

The strict female midgut-specificity and stage-specific inducibility of Gal4 drivers will provide powerful system to study hormone regulation, blood digestion and pathogen development in the midgut.

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4.7 Figures and tables

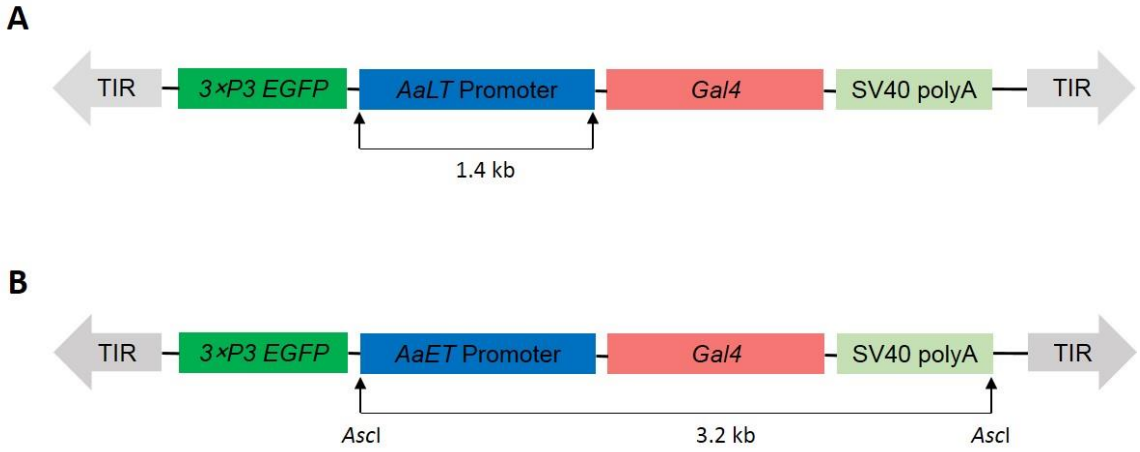

Figure 4.1. Schematic representation of the AaLT-Gal4 and AaET-Gal4 cassette (A). The AaLT-Gal4 cassette contains the *piggyBac*TIR sequences and the 5' regulatory region of the *AaLT*gene which is placed upstream of the *Gal4*. *EGFP* marker gene iscontroledby the eye-specific *3×P3* promoter. (B) AaET-Gal4 cassette consistsof Eye-specific selectable markers, EGFP.*Gal4* gene is placed downstream of *AaET* promoter region.

Chapter 5: Conclusions

5.1 Conclusions of the Thesis

This dissertation establishes 1) blood-meal-induced, midgut- and female-specific CP-Gal4 driver lines 2) SERCA is a direct target of aae-miR-275 and 3) two novel midgut-specific Gal4 drivers (under development).

Utilization of the 1.1-kb 5' upstream region of the carboxypeptidase A gene (CP) was demonstrated to lead a tissue-, sex- and stage-specific expression of the EGFP reporter in the midgut of CP-Gal4>UAS-EGFP mosquitoes after blood-meal. Insulin and Target-of-Rapamycin (TOR) have been proved to play an essential role in the *in vivo* knockdown and *in vitro* organ culture experiments, in which the EGFP reporter was activated in isolated midguts of previtellogenic CP-Gal4>UAS-EGFP females incubated in the presence of insulin. This effect was inhibited by knockdown of insulin receptor, or TOR specific inhibitor rapamycin. It is reported that expression of AaLT was regulate by insulin and TOR signaling pathways (Gulia-Nuss et al., 2011). These results showed that late phase digestive enzymes may share a same regulation mechanism.

After the CP-Gal4 driver line was identified, CP-Gal4>UAS-miR-275 Decoy hybrid lines were generated, which reduced aae-miR-275 (miR-275) level specific in midgut. Bryant et al. reported that inactivation of miR-275 by antagomir resulted in severe defects (Bryant et al., 2010). The expression analysis showed clearly that in hybrid midgut, decoy of miR-275 did reduced the level of miR-275, at the same time, down-regulated the transcript level of *SERCA*. Evidence like luciferase assay exhibited us direct

binding of miR-275 on *SERCA* 3'UTR. Injection of miR-275 mimic evaluated the *SERCA* mRNA level in both wild-type and hybrid midguts, and this mimic successfully overcame the defect brought by miR-275 Decoy. In compare with CP-Gal4>UAS-miR-275 Decoy hybrid lines, knockdown of *SERCA* or application of inhibitor TG displayed a very similar phenotype in wild-type mosquitoes. The Next step we will ascertain whether reduction of *SERCA* level in midgut provide an explanation for the disruption of blood digestion. Actin staining will be performed to demonstrate the muscle contraction ability is affect by reduced *SERCA* level. And western blot will be employed to detect protein levels of several important digestive enzymes.

In the last portion of this dissertation, we expect to utilize regulatory regions from two distinguish genes-AaET and AaLT to generate transgenic Gal4 driver lines, in which transgenes will be driven according a temporal- and spatial-specific expression pattern in *Ae. aegypti* mosquitoes.

5.2 References

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