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The Importance of Mosquito miRNA-275 in Blood Digestion and Egg Development

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

Master of Science

in

Entomology

by

Warren Paul Macdonald

March 2013

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The Thesis of Warren Paul Macdonald is approved:

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Committee Chairperson

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The Importance of Mosquito miRNA-275 in Blood Digestion and Egg Development

Chapter 1. Background and Significance

The yellow fever mosquito, *Aedes aegypti*, is a worldwide pest known for its ability to transmit a number of diseases including dengue fever, yellow fever and chikungunya fever. Dengue virus (DENV) and yellow fever virus (YFV) are tremendous global health threats and are considered highly pathogenic to humans (Gould and Solomon 2008). There are an estimated 2.5 billion people at risk of developing dengue fever and the more severe dengue hemorrhagic fever since 40% of the world population lives in high risk areas (http://cdc.gov). Though usually restricted to tropic and sub-tropic regions including Africa, Southeast Asia, Central and South America, there is concern that the virus can spread into more temperate regions. Dengue fever outbreaks have already occurred in the United States along the Texas-Mexico border (Brunkard et al. 2007) and there is speculation that as the global temperature increases so will the spread of DENV, due mostly to an increase in possible mosquito habitats (Degallier et al. 2010; Hsieh and Chen 2009; Martens et al. 1997).

Anopheles gambiae, also known as the malaria mosquito, is best known for harboring the most infectious form of the malaria parasite, *Plasmodium falciparum*. According to a global estimate from The World Health Organization (WHO), 216 million people were infected with malaria parasites in 2010, resulting

in 6,55,000 deaths (WHO, 2011). Although 81% of these desiease cases occurred in Sub-Saharan Africa alone, any area where *An. gambiae* mosquitoes reside, including the US, is at risk of reemergence. Since mosquito populations are becoming resistant to currently available insecticides (Montella et al. 2007), new control approaches and methods need to be developed to prevent further spread of disease.

Reprodutive biology of female mosquitoes

Anautogenous mosquitoes, such as *Ae. Aegypti* and *An. Anopheles*, have females that require vertebrate blood to produce eggs. As a consequence, these mosquitoes are vectors of numerous pathogens of devastating diseases. If steps are made to fully understand the reproductive biology of this mosquito, then new approaches to prevent the spread of arboviruses can be achieved. An angle of mosquito control, which has lots of potential, is disruption of the egg developmental cycle. The greatest physiological changes that a female mosquito goes through as an adult, such as peaks in juvenile hormone and ecdysone levels occur in conjunction with the egg cycle. Targeting the egg cycle of a vector for control could greatly reduce insect numbers by rendering females sterile. Some areas of the egg cycle process in *Ae. aegypti* have had much light shed upon them, but there are still many unknowns. Vitellogenesis, the important egg yolk protein deposition process and integral step in oogenesis, has been well defined in recent years. During vitellogenesis yolk protein precursors

(YPPs) are secreted by the fat body after a blood meal and are taken into the oocyte via receptor mediated endocytosis (Raikhel & Dhadialla, 1992). The initiation of vitellogenesis depends upon the cooperative action of multiple pathways including target of rapamycin (TOR) and 20-hydroxyecdysone (20E) (Raikhel et al., 2005). Knowledge of vitellogenesis has already been used to produce transgenic mosquitoes that can boost immune defense genes when vitellogenesis is initiated post blood meal (Shin et al. 2002; Bian et al., 2005; Antonova et al., 2009; Kokoza et al., 2010). The role of the insect steroid hormone 20-hydroxyecdysone (20E) in mosquito vitellogenesis and egg maturation has been elucidated at the molecular level, but what is still considered a “black box” is events occurring physiologically and developmentally after vitellogenesis to prepare the mosquitoes body for an additional clutch of eggs (Raikhel et al., 2005). Discovering the pathways and genes involved with this post-vitellogenic process would yield greater understanding of the egg cycle process and knowing more about the basic biology of a disease vector can lead the way for future control methods.

Digestion of blood in mosquitoes

The process which determines where an imbibed meal will go begins with identification of the liquid by sensory papillae located on the mosquito mouthparts. When blood is imbibed it is directed straight to the midgut, but the presence of certain sugars, such as in plant nectars, causes the liquid to be

diverted into the ventral diverticula or crop (Clements 2000). Upon procurement of a blood meal mosquitoes go through a series of phases in the midgut with major changes occurring in cellular morphology, organelle production, and synthesis and release of digestive enzymes (Clements 2000). During the first few minutes after a blood meal is obtained erythrocytes (red blood cells) are clumped together in the center of the midgut, leaving a thin space of cell free serum between the cell mass and the midgut epithelium. Within this space the peritrophic matrix (PM) will begin to assemble. The peritrophic matrix is a net-like semi-permeable chiten-containing protein layer secreted by the midgut epithelial cells to compartmentalize the midgut contents (Peters et al., 1992). The midgut lumen containing the ingested blood is referred to as the endoperitrophic space and the area between the PM and the epithelium is known as the ectoperitrophic space (Fig. 1). Digestive enzymes such as trypsin are most active in the ectoperitrophic space where is has been postulated that they traverse the pores of the PM and attack proteins at the periphery of the blood mass (Graf and Breigel, 1982; Borovsky, 1986). Digestion proceeds from outside of the blood mass inwards, and by about 24 hours post blood meal (PBM) the blood bolus appears black or brown as opposed to the bright red hue of freshly imbibed blood. Though digestion time is affected by temperature and humidity, most mosquitoes will have completed the digestion process and voided the midgut by 36-48 hours PBM (Briegel and Lea, 1975; Irby and Apperson, 1989).

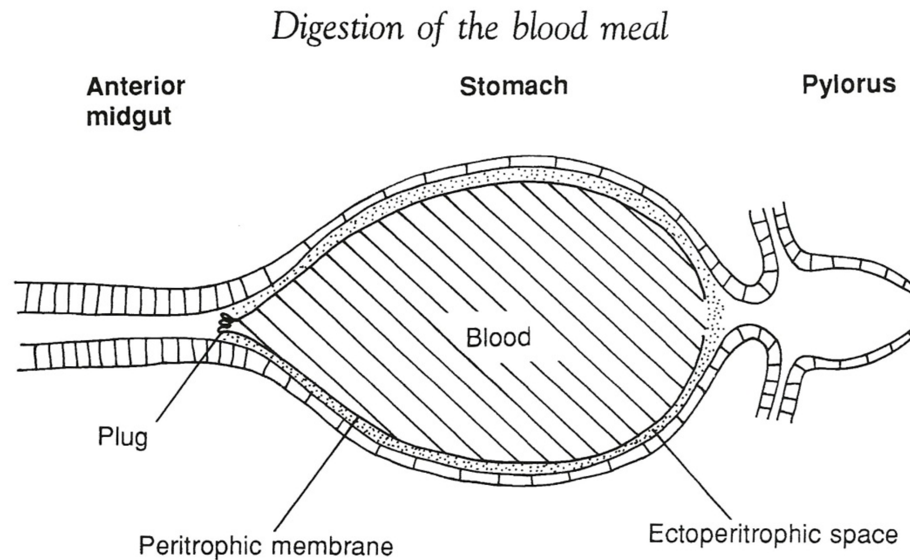

Fig. 1. Midgut during blood digestion. Diagram of a section throught he midgut of a femal mosquito during the course of digestion of a blood meal (Clements 2000).

In addition to blood digestion, the peritrophic matrix is thought to help with resistance to pathogens, toxins, and physical damage from the food bolus (Pascoa et al. 2002; Kato et al. 2002). Understanding the role of the peritrophic matrix is important because the midgut is the only surface continuous with the environment that is not covered in protective cuticle. This makes the PM a potential target for insect control and vector competence studies.

microRNA biogenesis

MicroRNAs (miRNAs) are 22-24 base pair small RNAs that are generated from hairpin like transcripts. It has been unveiled that the major role of miRNAs is inhibiting translation of mRNAs by binding to the 3'untranslated region (UTR) of target genes. The mechanisms by which miRNAs are processed are becoming

better understood (Fig. 2). After being transcribed by RNA polymerase II the miRNAs go into an initial hairpin structure called pri-miRNA while still in the nucleus. It is then cleaved by a Drosha complex and transported outside the nucleus by Exportin-5 as a pre-miRNA. One final cleavage by Dicer takes off the hairpin and leaves the miRNA in a mature duplex form of which one strand is separated and loaded into the RNA-induced silencing complex (RISC) while the other 'passenger' strand is usually degraded. The RISC then seeks out target mRNAs and either degrades or inhibits them through binding effectively, preventing translation by a great amount (Cullen 2004; Kim 2005). This type of post-transcription gene silencing is a natural occurrence of gene regulation.

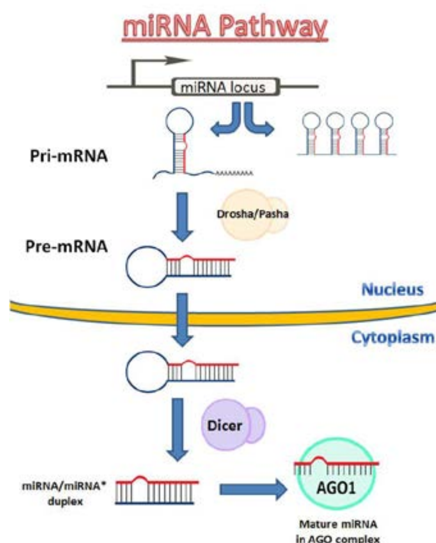

Fig. 2. Canonical miRNA pathway. miRNAs are transcribed as long transcripts resulting in pri-miRNA. This is cleaved by Drosha to produce pre-miRNA. This is exported out of the nucleus where Dicer cleavage occurs resulting in a miRNA/miRNA* duplex. The leading strand is then incorporated into an Ago1 complex.

<u>Functions of miRNAS</u>

The first discovered and most characterized miRNAs are lin-4 and let-7 from *C. elegans.* Both miRNAs are known to regulate timing of larval development

through binding of their specific mRNA targets of genes *lin-14*, *lin-28*, *lin-1* and *hbl-1* (Lee et al. 1993). Though the discovery of regulation between lin-4, let-7, and their targets may have seemed minor at the time, it eventually paved the way for all miRNA and target identification studies.

The roles of miRNAs in molecular processes and their target genes are being discovered more frequently than ever. It has been found that miRNAs take up a substantial ~1% of the genome and over one third of all mammalian mRNAs are controlled by miRNAs (Bartel 2004; Farh 2005). Studies have shown that miRNAs have important regulatory roles in many pathways including control of developmental timing, apoptosis, organ development and cell proliferation (Chen et al. 2004; Brennecke et al. 2003; Johnston et al. 2003). Apart from normal processes, miRNAs have also been linked to diseases and cancer. This had led miRNAs to be considered potential therapeutic targets for human disease (Caldas & Brenton 2005).

Potential targets of miRNAs are being revealed in a variety of diverse pathways, but many seem to be involved in development. It was found that miRNAs can be tissue specific when now well-known miRNA *bantum* induced suppression of proapoptotic gene *hid* and stimulated tissue formation during *Drosophila* development (Brennecke et al. 2003). Further research also showed that miR-9a suppresses apoptosis during *Drosophila* wing development and suppression of its activity causes a notched adult wing (Bejarano et al. 2010). Temporal

specificity of miRNA-138 was shown in zebrafish when the absence of the miRNA between 24-34 hours post-fertilization caused disrupted ventricular cardiomyocyte morphology (Morton et al. 2008). Through knockdown of a single miRNA CO_2 receptors in *Drosophila* that are normally localized to the antennae were found to also develop on the maxillary palps (Cayirlioglu et al. 2008). This developmental shift is even more significant because it creates an evolutionary intermediate with mosquitoes, which bear all their CO2 receptors on the maxillary palps.

The miRNA regulation of mouse angiogenesis has been shown through RNA interference knockdown of the gene for pre-miRNA cleaver Dicer, and manipulation of miR-92a (Bonauer et al. 2009; Yang et al. 2005). In the Dicer experiment no mouse got past the 14th day of gestation so it is apparent that miRNAs are an essential part of development. Blood vessel formation (specifically on the yolk sacs) was also impaired in the examined embryos compared to controls. Bonauer et al. 2009 found miR-92a to be a negative regulator of blood vessel formation by discovering underdeveloped blood vessels in miR-92a over expressed mice and less ischemic mice with the use of an antisense antagomir. Using the available tools to regulate miRNA numbers *in vivo* can both help visualize a phenotypic response and identify the target genes of the miRNAs. These distinct examples of temporal and tissue-specific

utilizations of miRNAs show a potential for reduction in non-target and pleiotropic effects when using miRNA antagomirs or over-expression.

Known *Drosophila* miRNAs are being tested for presence in mosquitoes and many of them are perfectly conserved. Skalsky et al. 2010 found 60 conserved and 7 novel miRNAs in *Ae. albopictus* and *Culex quinquefasciatus* compared to *Drosophila*. The presence of over 100 conserved miRNAs in *Bombyx mori* was confirmed with the majority expressed exclusively in specific tissue types (Liu et al. 2009; Liu et al. 2010). By using 454 sequencing 86 distinct miRNAs were uncovered in *Ae. aegypti*, 8 of which are novel (Li et al. 2009). Though the presence of these miRNAs is being confirmed their actual function is still unknown.

There have been some instances of miRNAs being associated with oocytes that have been noted so far. miRNAs have been found in early stage embryos of *Drosophila* before the start of zygotic transcription (Reich et al. 2009). This means that the miRNAs had to be synthesized by the mother and placed into the embryo. A novel miRNA was found to regulate cyclin B, an important protein in oogonial proliferation and oocyte meiotic maturation, in the Chinese mitten crab, *Eriocheir sinensis* (Fang and Qui 2009). Very few miRNAs have actually been shown to regulate the egg developmental cycle in any animal, so studying expression levels of candidate miRNAs is a step in the right direction.

Identification of miRNA targets

Though some miRNA roles have been determined, the majority of miRNAs that have been discovered lack known mRNA targets. This means that a great number of ways to regulate important pathways remains untapped and taking initiative during this time to find these miRNA targets could yield novel molecular pathway information. With this information one could pursue anything from new ways to combat disease to novel pest management applications (Chen et al. 2004). Because the ability to identify miRNA targets would be very useful, many techniques are being developed to carry out this common goal. Though there are a range of approaches, the types of methods currently being used fall into two categories: bioinformatics prediction, and molecular techniques. Both are beginning to give much needed insight into possible targets, but there are still almost no definite conclusions being drawn.

Bioinformatics Target Prediction

Analysis of the first known miRNA genes showed evidence of important pairing occurring between nucleotides 2-8, known as the “seed sequence” (Bartel 2009). Algorithms made to locate miRNA targets are based primarily on recognition of these seed sequences. Some features which also weigh heavily in these algorithms are evolutionary conservation, free energy, and nucleotide sequence motifs surrounding the seed sequence on the mRNA. TargetScan, one of the first available algorithms, requires an exact match for the seed region, while

Pictar allows mismatches under the condition they meet a stringent free energy cut off (Krek et al. 2005). The miRanda algorithm also puts focus on the seed sequence but allows for seed G-U wobbles (not Watson-Crick base pairing) and bulges which allow the program to correctly predict *lin-41* as a target of *let-7*, a well-known miRNA-mRNA pair, in *C. elegans* (John et al. 2004, Vella et al. 2004). Though useful, many algorithms that focus on the seed sequence are under scrutiny due to an overwhelming number of false positives generated (Alexiou et al. 2009). The prediction program rna22 takes an alternative approach by using lowest free energy of the overall heteroduplex as its primary focus and completely throws out evolutionary conservation. This variation of bioinformatic prediction has shown a low false-positive output, but seed-based prediction still has the greatest specificity and sensitivity (Miranda et al. 2006, Sethupathy et al. 2006).

When using bioinformatics prediction programs there are a number of drawbacks that need to be taken into account. An annotated sequence database is needed in order to use one of these algorithms, and when multiple databases are available there can be a vast difference in positive UTRs solely based on variances in annotation. The tendency of algorithms to overestimate targets might stem from their inability to account for the tissue and temporal specific nature of miRNAs. Astoundingly, even with the great amount of false-positives, some biologically important miRNA targets are still missed. A recent study

suggests that up to 45% of miRNA/target associations my lack a perfect seed match (Zisoulis et al. 2010), meaning prediction programs may need to shift focus from the seed region or add additional guidelines which can incorporate newly discovered pairing tendencies. In their current state, bioinformatic prediction programs do not have the reliability to me more than just guides that may help point researcher in seemingly correct direction.

Genetic Approaches and Expression Analysis

Approaching the identification of miRNA targets via molecular techniques seems to address many of the gaps left by algorithms, but leaves other questions to consider. If a specific miRNA is of interest, then reverse genetics can be employed through specific knockdown or over-expression. The use of miRNA antagomirs to bind specific miRNAs in *vivo,* can be a useful tool and resulting phenotypes may give hints to possible target genes (Krutzfeldt et al 2005). Forward genetics can also be used to gain insight on a particular miRNA. One study did a genome wide screen to look for genes whose knockdown result matched that of miR-19 over-expression (Mavrakis et al. 2010). Of eight candidates identified four were found to be true gene targets.

Though it is possible to gain insight on possible targets via a phenotypic reaction, comparison of transcriptomic data to a miRNA over-expression experiment is even more useful. Through microarrays or high throughput sequencing it can be

deduced which genes are being highly up-regulated (in the presence of antagomirs) and down-regulated (in over-expression). A similar approach can be taken when dealing with proteomics, but instead of checking transcript levels, stable isotope labeling with amino acids (SILAC) can be used to check for overall changes in protein levels under the two treatments. (Selbach et al 2008).

It should be noted that although these target detection methods can be specific to tissues and time points, they cannot distinguish direct or indirect targets. A great wave of changes can occur when a miRNA levels are manipulated, and transcriptome and proteome analysis can only see the overall changes whether they be a direct result or downstream.

Immunoprecipitation of Ago

The miRISC, which carries out the search for target mRNAs and subsequently silences them, consists of the mature miRNA guide strand and a number of proteins including Argonaut (Ago). The Ago protein, which holds the encased miRNA, has been the most common target to be used in immunoprecipitaion of the miRISC. By either tagging Ago or using an Ago antibody possible miRNA target information can be obtained by sequencing the associated mRNAs which are still intact. Biochemical pulldown of miRISC components can only be for all miRNAs currently in the tissue simultaneously, it cannot directly identify mRNAs for a specific miRNA. Attempts at biotinylating specific miRNAs to narrow the

search to exact match mRNAs have not yet worked and result in non-specific binding of ribosomal mRNAs (Orom & Lund 2007, Orom *et al.* 2008).

Recently developed methods HITS-CLIP (high throughput sequencing by crosslinking and immunoprecipitaion) and PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipiation) have the ability to crosslink both the miRNA and the mRNA with Ago. While both methods have been showing a low occurrence of false-positives and false-negatives they also very technically challenging and consist of several steps. Even so, they have thus far given much insight into 3' UTR binding locations and non UTR miRNAs binding (Hafner et al. 2010).

Validation

Conclusions of miRNA-gene regulation pairs cannot be solely made by any of the previously described methods alone; individual validation of putative targets is imperative. A number of methods can be employed to validate candidate genes including; showing in inverse relationship between the protein and the miRNA, using a luciferace or other reporter assay with the entire 3' UTR of the gene in question, and mutation or deletion of the miRNA binding site leading to renewed gene expression.

The lab proposed to find miRNAs significant to the egg cycle process in *Aedes aegypti* and to test how they interact with vitellogenesis and 20E levels. miRNA antagomirs can be used to effectively silence miRNAs *in vivo* by inhibition (Krutzfeldt et al. 2005). By using this method we can confirm which miRNAs and *Aedes* genes are involved in regulation of the egg cycle process. Once these miRNAs are identified as egg cycle regulators, further gaps in knowledge can be filled by uncovering their multiple gene targets.

By screening recently tested *Drosophila* miRNAs that are up-regulated during key physiological events, such as when 20E levels are high, Dr. Bart Bryant, a Post-Doctoral researcher in the Raikhel lab, has found two possible miRNA candidates that might have a part in the egg cycle process (Fig. 3). Up-regulation of miR-275 and miR-305 in the mosquito fat body, the center of vitellogenesis, correlates with a 20E peak in the female mosquito indicating their possible roles in vitellogenisis and egg cycle maintenance. Along with Dr. Bryant, I began characterizing the roles of miR-275 in *Ae. aegypti* through its specific knockdown. My personal work is a continuation of research into miR-275, exploring its downstream effects and possible direct targets as well as preliminary studies of miR-275 in *An. gambia*e.

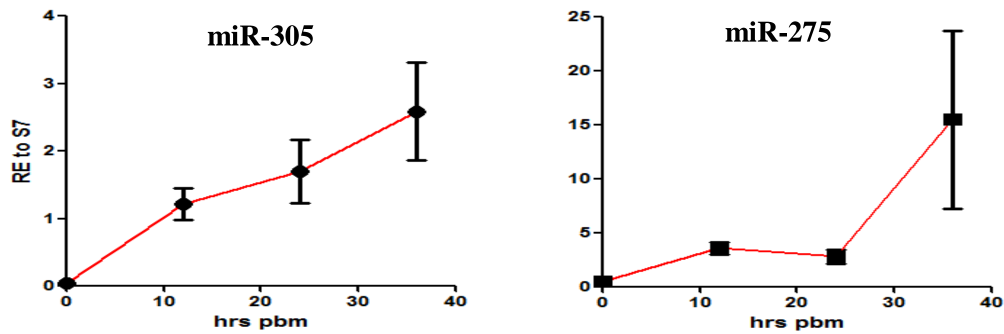

Fig. 3. miRNA expression trends in the mosquito fat body during vitellogenesis. Expression analysis for miR-305 and miR-275 at different time intervals post blood meal. Expression levels show an upward trend near the end of vitellogenesis. This experiment was performed by Dr. Bart Bryant (Bryant et al. 2010).

By antagomiR directed specific knockdown of miR-275 we will be able to see what effect, if any, the absence of miR-275 will have on the egg development process of important mosquito species. Because miR-275 has a delayed induction, but is up-regulated in a similar pattern as important regulatory hormone 20E some degree of development complication should occur in its absence. As it turns out, when miR-275 was knocked down deficiencies in both blood digestion and egg development arose. Using the miR-275 knockdown phenotype as a hint to its function, I propose to investigate the role of this miRNA by commencing a search for its target genes and testing candidates with molecular techniques.

Chapter 2. Materials and Methods

Animals

Aedes aegypti mosquitoes from the UGAL/Rockefeller strain were raised as described previously (Roy et al. 2007). Female mosquitoes 3–5 d after eclosion were fed on the blood of anesthetized white rats to initiate egg development. *Anopheles gambiae* NGS mosquitoes, obtained from Dr. Bradely White, were colonized from N'Goussa, Camaroon in 2008. The mosquitoes were reared as follows: **Day 1:** Eggs are by allowing them to dry for 5 mins while they sit on the sides of the tray. After hatching 200 larvae are dispensed into a wax paper cup. A tray is filled with 1 liter of D.I. water and the larvae are poured in. Larvae are fed the required number of scoops. **Day 2 – Day 6:** Larvae are fed the required number of scoops. **Day 7 – Day 8:** Pupare are collected and dispensed in a new cup. Cup is placed in an adult mosquito cage along with 2 cups of sucrose soaked cotton balls. *An. gambiae* female mosquitoes were blood fed in the same mannar as *Ae. aegypti* except feeding was performed in the dark.

mRNA and miRNA Expression Analysis

Ae. aegypti and *An. gambiae* miRNA sequences available at miRBase (http://www.mirbase.org/index.shtml) were examined for their location in the genome using BLAST searches at the Broad Institute site (http://www.broadinstitute.org/annotation/genome). miRNA expression was analyzed as described previously (Bryant et al. 2010).

First, RNA was isolated by TRIzol (Invitrogen) extractions from fat bodies and midguts of blood-fed female mosquitoes at various time points. RNA was then digested with DNase I (catalog no. 18068015; Invitrogen) and subjected to cDNA production with the miScript reverse transcription kit from Qiagen. cDNA obtained from this was then subjected to expression analysis with the miScript SYBR Green PCR kit from Qiagen. The PCR condition was as follows: Step 1, 95 °C for 15 min; Step 2, 94 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s for 50 cycles; Step 3, 95 °C for 1 min. This was then followed by melt curve analysis. The miScript PCR System was also used to analyze IMUC1 and APER-50 transcripts using S7 as an internal control. Expression data were plotted using 2-ΔCt whereby the cycle threshold (Ct) for the gene of interest is compared with the Ct of the internal control gene, in this case S7 (Schmittgen et al. 2008).

Synthesis and Application of AntagomiRs

Antagomirs were obtained from Dharmacon using the custom RNA module at http://www.dharmacon.com/rna/rna.aspx. Antagomir to miR-275 was 5′ mC* mG* mC mG mC mG mC mU mA mC mU mU mC mA mG mG mU mA mC mC* mU* mG* mA* mA 3′. The control antagomir termed missense was 5′ mC* mG* mC mU mU mU mC mG mU mG mG mU mU mC mU mG mG mU mA mC* mC* mU* mU* mA 3′. A PS backbone instead of the usual PO backbone is indicated by a “*” . “m” is an OCH3 group on the 2′ end of the base instead of the usual OH group. A 3′ cholesterol group added to each RNA oligo for potency and longevity reasons (Förstemann et al. 2007). Antagomirs were constructed as outlined in

Horwich and Zamore 2008. Female *Ae. aegypti* mosquitoes were injected at a dose of 100-200 μM at a volume of 0.5 μL per mosquito. *An. gambiae* mosquitoes were injected at a dose of 200 μM at a volume of 0.25 μL per mosquito because of their smaller body size.

Actin Staining of Midguts and DNA Staining of Oocytes

For fluorescence staining of midguts, mosquitoes were intrathoracically injected with and allowed to incubate for 30 min in a solution containing 0.165 μM phalloidin Alexa Fluor 488 (A12379; Invitrogen), 1% Triton-X 100, and 8% formaldehyde, whereby APS (Aedes physiological saline) was the diluent according to conditions detailed by Glenn et al. 2010. Midguts were then dissected and washed in APS-T (Aedes physiological saline with 0.3% Triton-X 100) and mounted using mounting media from VectaShield (H1000). For fluorescence staining of oocytes, mosquitoes were dissected and ovaries incubated in APS-T for 10 min. The ovaries were then put into a DNA staining solution containing 5 μM Hoescht 33342 (H1399; Invitrogen) in APS for 10 min. All imaging was performed using a Zeiss microscope, AxioObserver A1, and images were obtained using AxioVision software

Western Blot Analysis

Protein analysis of Aper-50 signaling was done according to ref. Shao et al. 2005. Briefly, midguts from blood-fed female mosquitoes were obtained 24 h PBM. These midguts were homogenized and lysed and run on Tris-Glycine gels

(Invitrogen) and transferred to PVDF membranes. These membranes were probed with Aper-50 antibodies according to ref. Shao et al. 2005 obtained from the lab of Marcelo Jacobs-Lorena.

RNA Interference

Gene models for Aper-50, IMUC1, and MBD-R2 in *Ae. aegypti* were prepared by Dr. S. Aliyari. For RNAi experiments, PCR products were cloned into the TOPO-TA vector from Invitrogen (catalog no. 45-0640) according to the manufacturer's specific instructions. PCR products were then used to make dsRNA with the MEGAscript kit from Ambion (catalog no. AM1334) according to the manufacturer's specific instructions. Mosquitoes were anesthetized with ice 1 to 2 d after eclosion and injected into the thorax at a volume of 0.5 µL with appropriate dsRNA molecules at ≈1.5–2 µg/µL. LacZ was used as a negative control. Mosquitoes were allowed to recover for 4 to 5 d before blood feeding.

RESULTS AND DISCUSSION

Chapter 3. Specific depletion of miR-275 in *Aedes aegypti* drastically affects both blood digestion and egg development. (Bryant et al. 2010)

Initially under the supervision of Dr. Bryant, I used a miRNA antagomiR depletion technique to knockdown miRNAs in mosquitoes. Utilization of this technique has an advantage of being able to deplete a specific miRNA. I tested the specificity of a custom made RNA oligo directed towards miRNA-275 from Dharmacon (http://www.dharmacon.com). Oligos contain some modification such as a PS backbone instead of the usual PO backbone and a 3' cholesterol group to extend longevity *in vivo.* There is a significant drop in only miR-275 expression and not in Bantam when the custom oligo directed towards miR-275 is injected, meaning the knockdown effect is specific to miR-275 (Fig. 4).

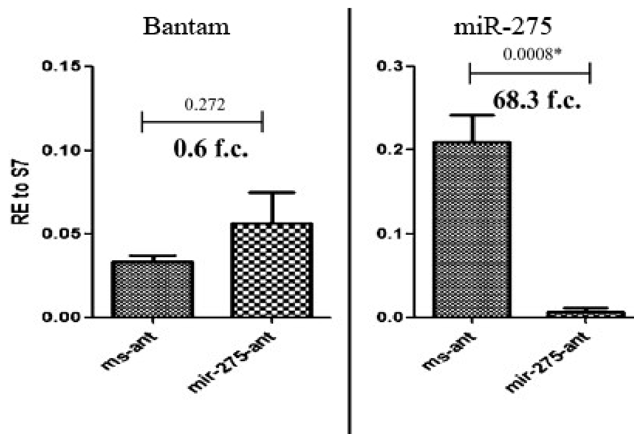

Fig. 4. Expression analysis of miRNAs Bantam and miR-275 in mosquito fat bodies 24H PBM. Bantam expression is not significantly changed between miR-275 antagomir injection and missense antagomir injection. However, there is a drastic depletion of miR-275 (68.3 fold change) after specific antagomir knockdown (Bryant et al. 2010).

After knockdown of miR-275 there were dramatic defects in intake and digestion of blood in mosquito females. This phenotype is characterized by a large volume of undigested blood in the crop and the presence of less digested or expelled

blood in the midgut (Fig. 5a). The crop in wild-type mosquitoes, used only to store nectar, is normally bypassed when the mosquito is taking a blood meal. This means that upon ingestion, blood passes through the esophagus and the midgut anterior portion straight into the stomach. In the stomach, the blood forms a bollus surrounded by a chitin-protein envelope called the peritrophic matrix, within which the ingested blood undergoes digestion (Shao et al. 2001; Lu et al. 2006; Isoe et al. 2009). Somehow in the miR-275-ant background, blood is being regurgitated into the crop and remains partially undigested in the midgut as well. At the same time point that the disruption in digestion was observed in the mir-275 knockdown mosquitoes (24 h PBM), the stomach of the wild-type mosquito females contained a compact dark brown bolus of digested blood (Fig. 5E). The midgut of the ms-ant control mosquito had a similar morphology (Fig. 5C). Examination of females from all backgrounds 3 h PBM showed no defects in digestion meaning that the miR-275-ant phenotypic blood is somehow being moved to the crop sometime after the blood meal. In addition, as shown on images with actin staining, the junction between the anterior midgut and the stomach was not as constricted in a female with the mir-275-ant background compared with those in the wild-type and the ms-ant mosquitoes (Fig. 5 B, D, and F). Under the supervision of Dr. Bryant I performed numerous knockdown trials, scoring and documenting mosquitoes with the mentioned phenotypes. I also performed the actin stains and all microscopy involved including light and fluorescent.

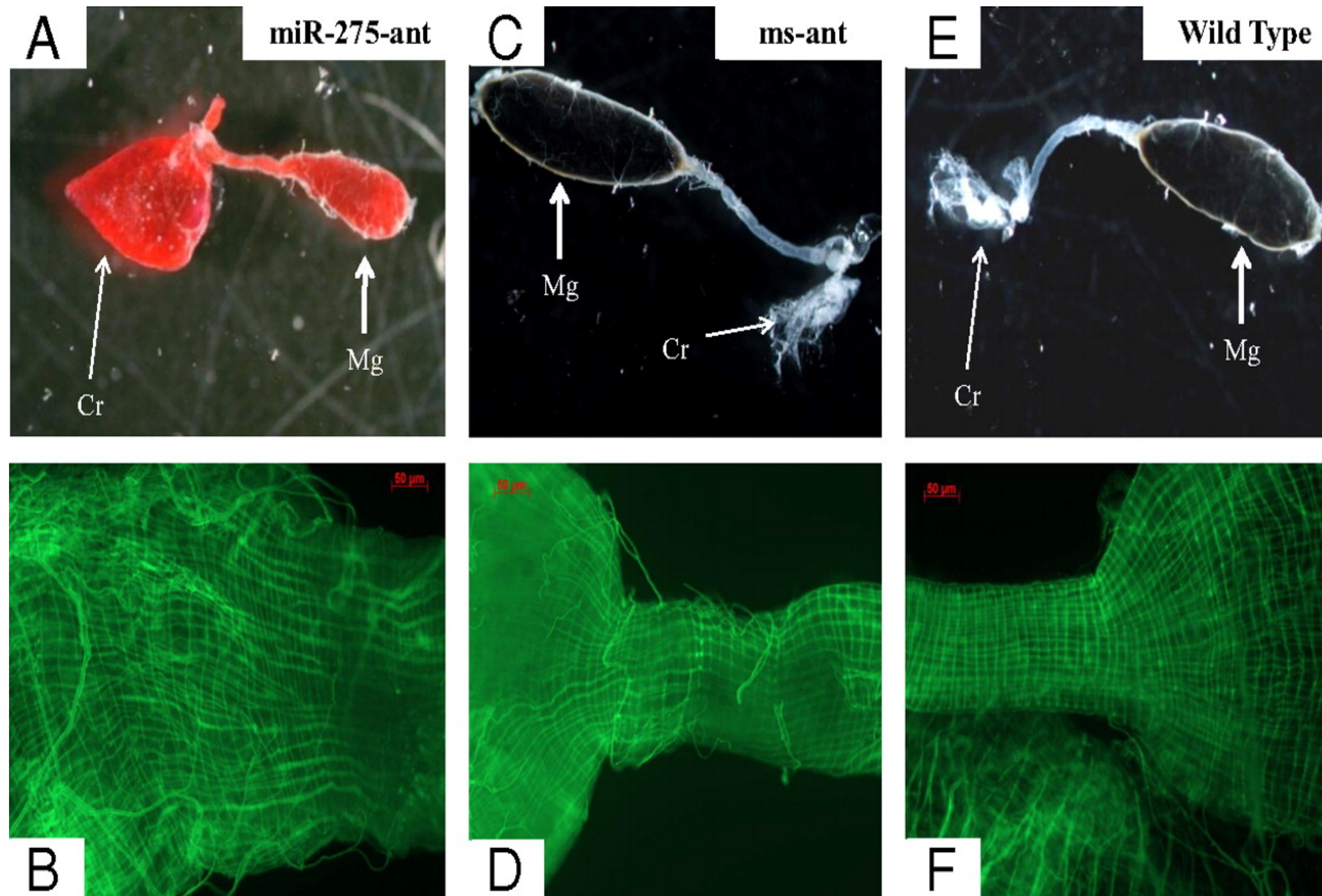

Fig. 5. Depletion of miR-275 drastically affects blood digestion. (A and B) miR-275-ant background; (C and D) ms-ant background; (E and F) wild-type background. Midguts were obtained 24 h PBM from different backgrounds and analyzed for blood digestion defects. A, C, and E are bright-field observations of the backgrounds, and B, D, and F are actin staining of the backgrounds.

Another developmental defect in mir-275-ant mosquitoes was inhibition of egg development. Normal mosquito ovaries are made up of about 150 ovariols, each of which consists of a primary follicle (egg chamber) and a germarium with an undifferentiated secondary follicle. During the pre-vitellogenic stage, primary follicles grow from 40 to 110μm and nurse cells occupy about three quarters of the follicle volume (Clements, 2000). After vitellogenesis is initiated upon blood meal, the oocyte grows exponentially by accumulating yolk protein precursors (YPPs). At this time the nurse cells shrink in volume and undergo cell death. At 24 h PBM wild-type and ms-ant mosquitoes had primary follicles averaging 222μm in length that were filled with yolk and very few visible remaining nurse cells at the apex of the follicle (Fig. 6 C, E). Egg development in mir-275-ant mosquitoes was severely compromised (Fig. 6 A). When examined at the same time point PBM, mir-275-ant background mosquitoes had substantially smaller ovariols (135μm on average) and nurse cells had not yet began diminishing (Fig. 6 A, B). After individual measurement follicle length was found to be significantly lower on an average in the miR-275-ant background compared to both controls (Fig. 7 B, $P<0.0001$). Another defect in the miR-275-ant background ovaries was a premature differentiation of secondary follicles which contained large nurse cells and a well-defined follicular epithelium (Fig. 6 B). In wild-type and ms-ant backgrounds there were no visible tertiary follicles, only undifferentiated secondary follicles with few small nuclei (Fig. 6 D, F).

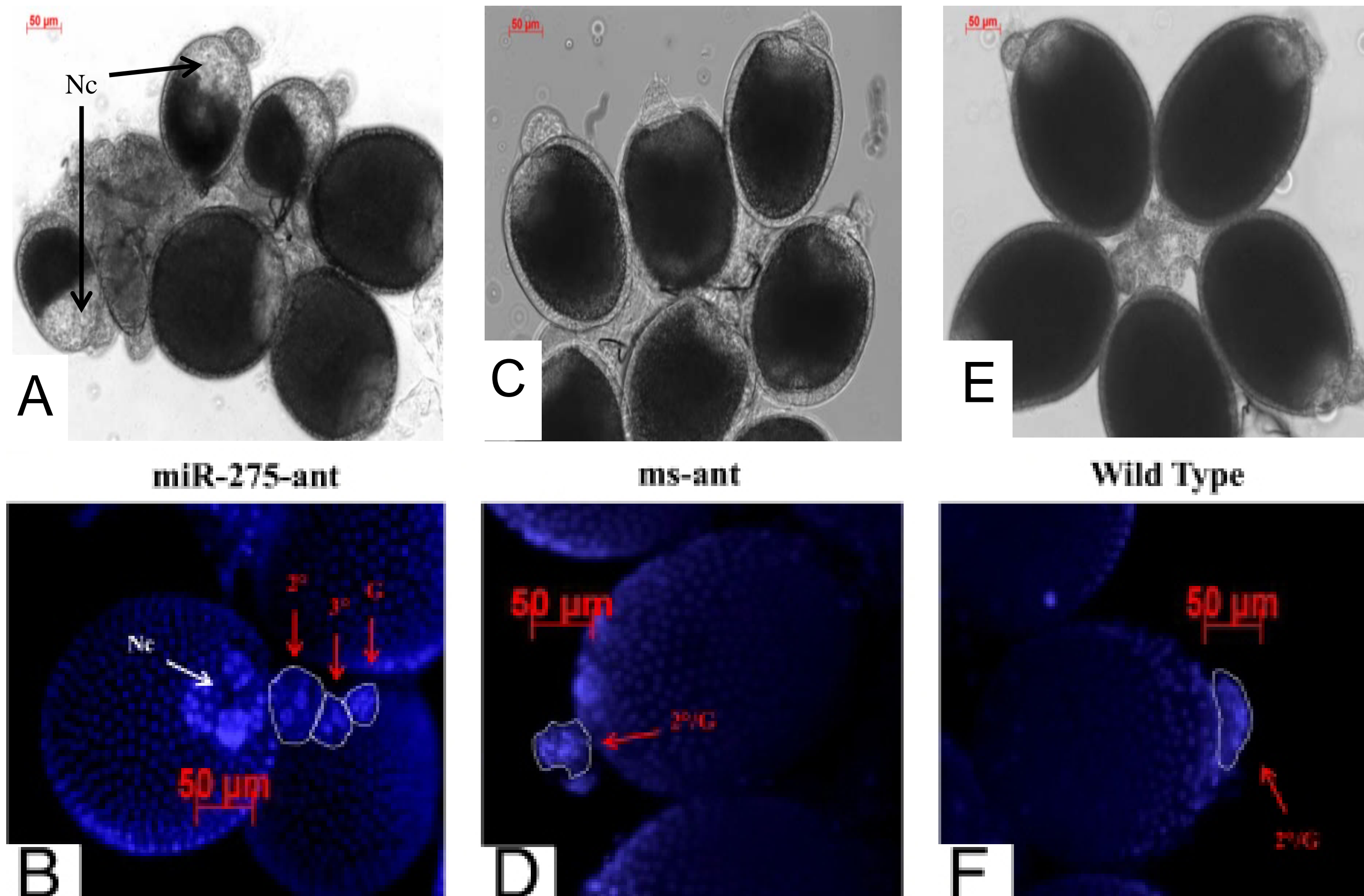

Fig. 6. Depletion of miR-275 drastically affects egg development. (A and B) mir-275-ant background; (C and d) ms-ant background; (E and F) wild-type background. White and black arrows point to nurse cells, and red arrows point to follicles. Nc, nurse cells; 2°, secondary follicle; 3°, tertiaryfollicle; 2°/G, secondary follicle within germarium. Ovaries were obtained 24 h PBM from different backgrounds. Photos were captured using brightfield (A,C, and E) and florescent microscopy after Hoescht nuclear stain. (Bryant et al 2010).

Because the sizes and developmental state of miR-275-ant background follicles vary, there is still some successful deposition of YPPs occurring. This means YPPs are still being produced in the fat bodies and vitellogenin (Vg) production is not completely compromised, if it is being affected by mir-275 knockdown at all.

Although the miR-275 depletion phenotype was very drastic, its penetrance was relatively low. Dr. Bryant and I have ran numerous experiments (over 25) and the resulting penetrance was an average of 30% to 40%. Despite the low penetrance the described phenotype has shown how important the role of a single miRNA can be in regulating key developmental events in mosquitoes.

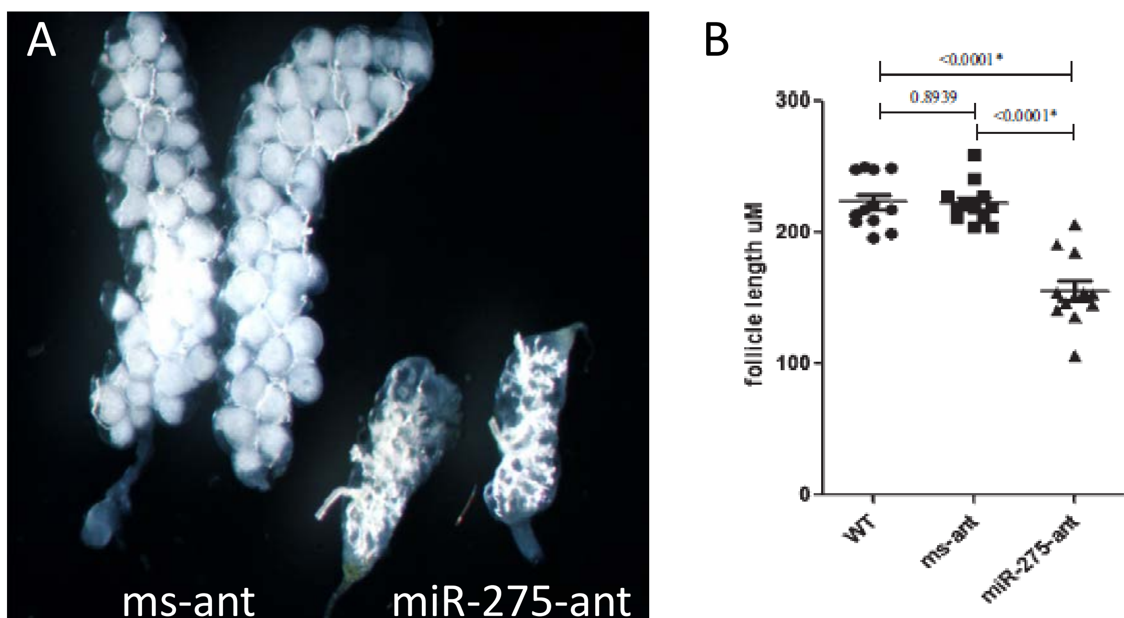

Fig. 7. Depletion of mir-275 drastically affects overall ovary size and follicle lengths. (A) Examples of the whole ovaries 24hrs PBM when injected with missense antagomir (ms-ant) and miR-275 antagomir (miR-275-ant). (B) Graph of individual ovariol follicle length from miR-275-ant injected and controls.

Conclusions from analysis of miR-275-ant background mosquitoes tell us that we have identified a miRNA affecting blood digestion with a potential to link to egg development. Since it is a possibility that the lack of egg maturation stems from the disrupted blood digestion, I wanted to do further research on why the blood was not being digested.

Chapter 4. The Effect of miR-275 Depletion on Peritrophic Matrix genes AeAPER50 and AeIMUC1

The presence of blood that has been both misplaced and undigested gives indication that the peritrophic matrix (PM), or lack thereof, could be contributing the phenotype. As mentioned previously, the main purpose of the PM is to contain and facilitate digestion of a blood meal. By testing for known protein components of the PM I could estimate its presence or absence under knockdown conditions of miR-275. Thus far two PM proteins have been characterized in *Ae. aegypti*, peritrophin 50 (Aper50) and mucin-like (IMUC1) (Shao et al., 2005; Rayms-Keller et al., 2000; Morlais and Severson, 2001). An expression profile was performed on both genes using midguts dissected at various hours PBM and newly designed primers. The real time PCR results (Fig. 8) showed steadily increasing expression of both genes after a blood meal. IMUC1 did have some expression prior to a blood meal as well.

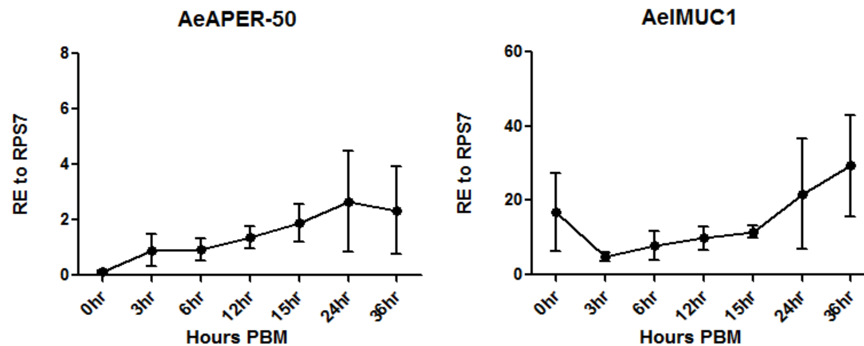

Fig. 8. Expression profile of peritrophic matrix genes APER50 and IMUC1 using midgut tissues.

Since both PM protein genes have a role in PM formation, we believed that knocking down transcripts of these genes should interrupt PM establishment and result in less or undigested blood. dsRNA probes were made against both PM genes and injected seperately into recently emerged adult female mosquitoes. A portion of the injected mosquito MGs were used to check for gene knockdown efficiency and the remaining mosquitoes were blood fed and dissected 12 hours later. The knockdown efficiency was substantial (Fig. 9), and when one gene was knocked down, transcripts from the other PM gene were also effected.

The phenotype observed was not as drastic as with the miR-275 knockdown (no blood in the crop), but a distinct lack of digestion could be seen when compared to controls. IMUC1 (Fig. 10 A,B) and APER50 (not shown) knockdown MGs appear more undigested and hence red compared to control (Fig. 10 C left and right). The brighter red color makes them appear similar to some of the MGs of miR-275 knockdowns (Fig. 10 C middle).

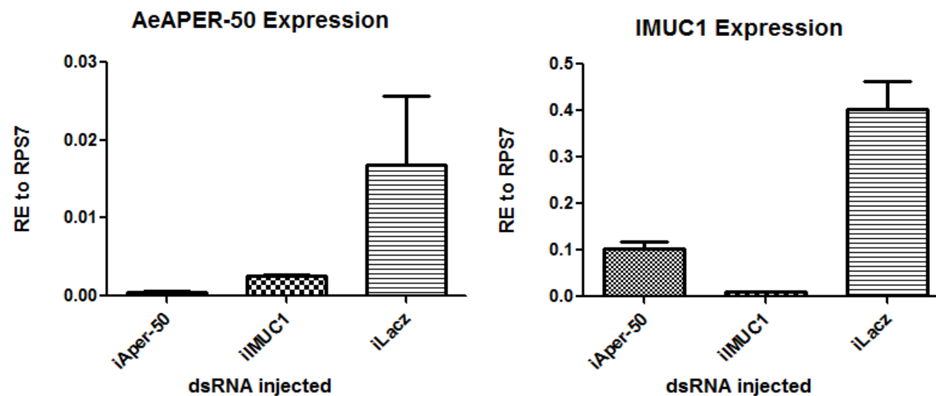

Fig 9. Relative transcript expression of PM proteins after PM protein knockdown via RNAi. The graph on the left shows APER50 expression in iAPER50, iIMUC1, and iLacZ backgrounds. The right graph shows IMUC1 expression in the same backgrounds.

The similarity in appearences between MGs of both PM gene knockdowns and miR-275 antagomiR knockdowns at 12 hour PBM implicates that there may be some connection between the two, either direct or downstream.

Protein levels were also assessed to see if transcript knockdown did lead to protein reduction. Preliminary resusts of a Western blot using antibodies against Aper50 shows protein levels are reduced in the same manner as transcripts (Fig. 10 E). No protein was present in Aper50 knockdown mosquitoes whereas normal amounts could be seen in controls. An additonal Western Blot analysis shows PM protein Aper50 levels are down in the positive phenotype midguts from miR-275 knockdowns (Fig. 11). This means that translation of PM proteins may be blocked in the MG while miR-275 is not present.

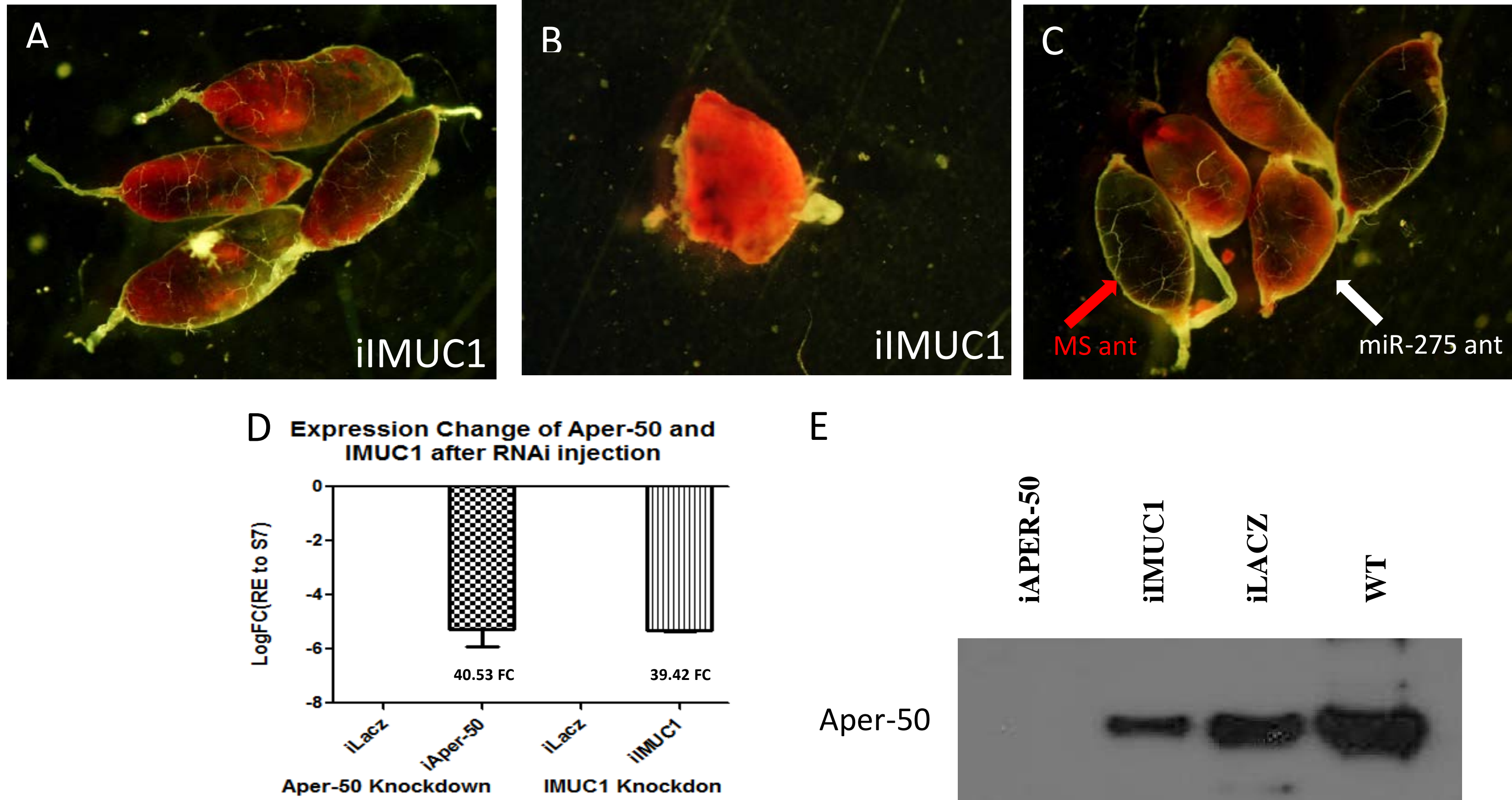

Fig. 10. Knockdown of PM proteins using RNAi. (A,B) MGs from a IMUC1 knockdown mosquito 12 hours PBM. (C) MGs from miR-275 antagomir injected and missense antagomir injected mosquitoes 12 hours PBM. (D) Log Fold change of IMUC1 and APER50 transcripts after knockdown. (E) Western blot using APER50 anti-body showing absence of protein in APER50 knockdown mosquitoes.

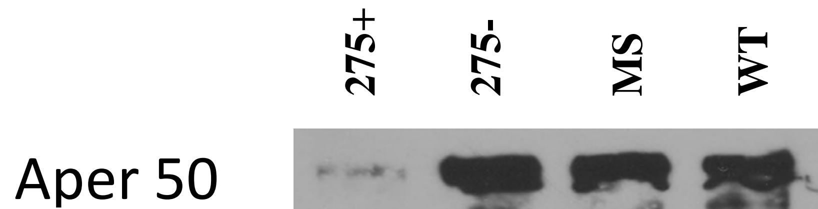

Fig. 11. Western Blot showing presence of PM protein APER50 in backgrounds of miR-275 knockdown. Levels of APER50 protein are greatly affected by miR-275 specific knockdown when the phenotype is present in MG 12hrs PBM. 275+; positive phenotype miR-275-ant background, 275-; negative phenotype miR-275 background, MS; missense-ant background, WT; wild type.

Lack of Aper50 protein in positive phenotype could also be explained by cases in which blood was expelled from the MG. With most of the blood not present less PM genes would be transcribed and translated, accounting for the drop in protein level of Aper50 in positive phenotype mosquitoes. Because the crop has the ability to contract and expel sugar liquids into the MG it may be performing this same action with regurgitated blood, sometimes giving the appearance of undigested blood in the MG 24hrs PBM.

Chapter 5. Specific depletion of miR-275 in *Anopheles gambiae* causes similar deficiencies in digestion and egg development

Following the same procedure outlined above, I specifically depleted *Anopheles gambiae* females of *miR-275* by means of the same antagomiR approach to study its role in blood digestion and egg development. This depletion resulted in similar egg maturation problems along with blood expulsion from the midgut (Fig.12).

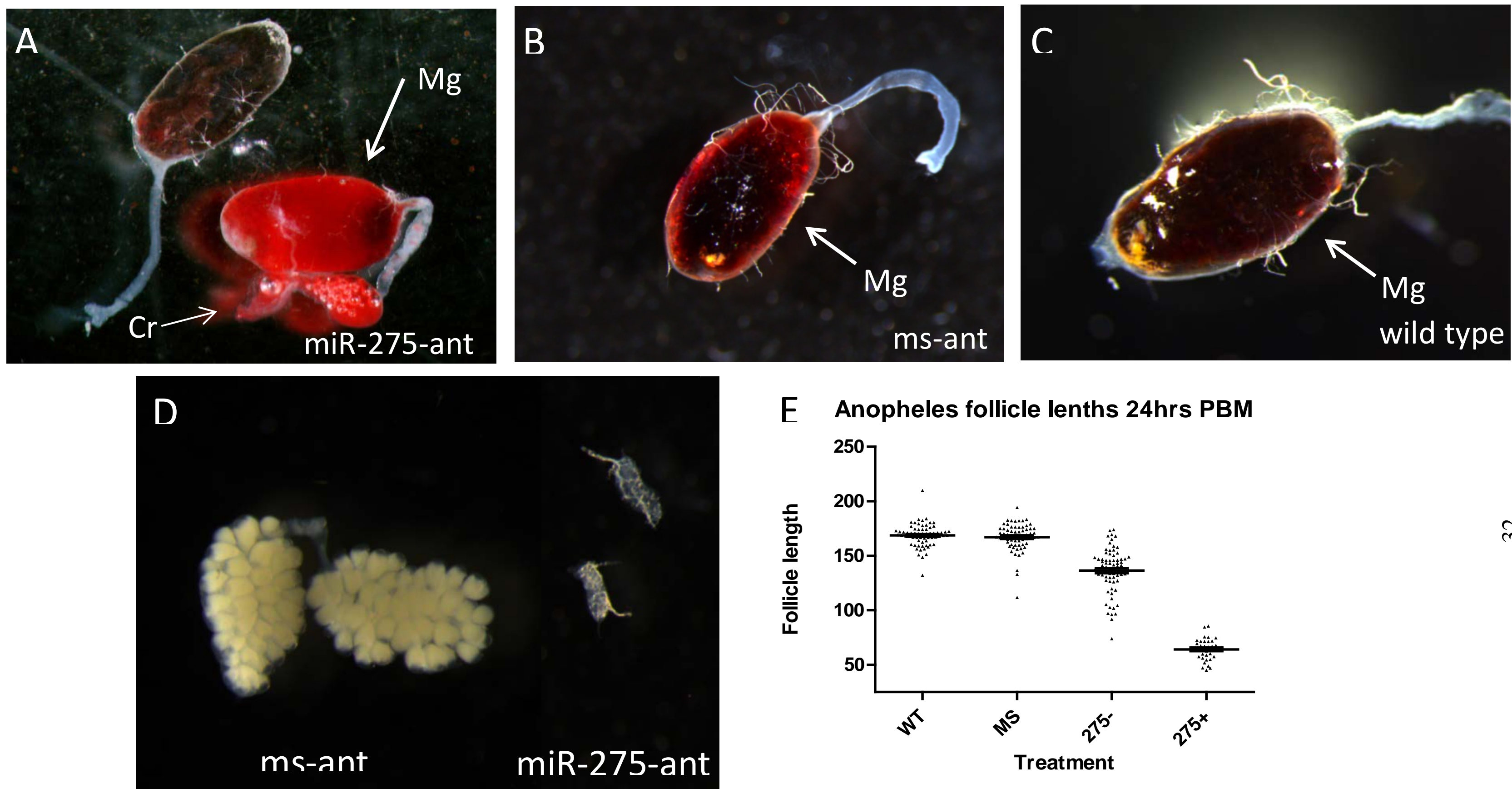

Fig. 12. Depletion of miR-275 drastically affects blood digestion and egg development in female *Anopheles gambiae* mosquitoes. (A) Crop (Cr) and midgut (Mg) in miR-275-ant background 24 hours PBM. Both a non-phenotypic and phenotypic example are shown; (B) Mg in ms-ant background; (C) Mg in wild-type background. (D) Ovaries of a control ms-ant background compared to an underdeveloped pair in miR-275-ant background. (E) Follicle length comparison of ovarioles 24hrs PBM. Significant differences were found between controls and both experimental treatments

A comparison of follicle lengths confirms the severity of the phenotype. Ovarioles from mosquitoes who expelled blood from their midguts in the absence of miR-275 (275+) had follicles less than half the length of controls on average (Fig. 12 E). Follicles measured from miR-275-ant mosquitoes which did not show expelled blood from the midgut (275-) still show a significant reduction in size. Though these results seem promising they are still preliminary and many more injection trials are necessary before any conclusive remarks can be made.

Chapter 6. A bioinformatics search for miR-275 targets has yielded candidate genes

Since the ultimate goal for miR-275 is to find its gene targets I took the first step by meeting with Dr. Juan Joval about a bioinformatics analysis of the *Aedes* genome. Dr. Joval decided to write his own program based off of the algorithm used in Grimson et al. 2007 that focused primarily on 'seed sequence' (nucleotides 2-8) pairing of the miRNA to sites in the 3' untranslated region (UTR) of coding genes. I had several meetings with Dr. Joval about what criteria and scoring we felt would help determine good candidate genes. We decided that in addition to a 6mer 'seed sequence' requirement, candidate genes would also rank higher if: there was perfect pairing of nucleotides 13-16, sites were within AU-rich regions, sites were within the first or last quartile of the 3'UTR, sites were not within the first 15 nucleotides of the UTR, and if there are multiple sites within the same UTR. This program, when used against the online

database of *Aedes aegypti* 3'UTR expressed sequence tags (ESTs) gave rise to a list of over 100 gene candidates. Of these, the candidates that have a total score of over 6 and at least 2 potential binding sites are shown (Table 1). I researched many of these genes for known protein biding domains and *Drosophila* homologs. We decided to focus only on the top hit because it had five probable binding sites all in the same UTR. The Drosophila homolog of this gene is called methyl binding domain 2 (MBD-R2).

ID	Length of UTR	Start	End	>15	Match 13-16	<1/4 or >3/4	UA Score	Sub-total	# sites	Total score
AAEL009416|AAEL009416-RA|941280|942789	1510	1233	1238	1	0	1	1.7587	3.7587	5	14.7532
AAEL001394|AAEL001394-RA|904506|906235	1730	1161	1166	1	0	0	2.4068	3.4068	2	8.4533
AAEL007883|AAEL007883-RC|1162481|1164041	1561	1246	1251	1	0	1	3.1400	5.1400	2	8.3161
AAEL005974|AAEL005974-RA|276116|276657	542	253	258	1	0	0	3.4354	4.4354	2	8.2658
AAEL014971|AAEL014971-RA|70850|72182	1333	307	312	1	0	1	2.0622	4.0622	2	7.9694
AAEL010784|AAEL010784-RA|365879|366960	1082	947	952	1	0	1	1.9153	3.9153	2	7.7486
AAEL005832|AAEL005832-RA|266314|268248	1935	1393	1398	1	0	0	2.6702	3.6702	2	7.6418
AAEL001850|AAEL001850-RB|586200|586685	486	350	355	1	0	0	3.7713	4.7713	2	7.0583
AAEL013250|AAEL013250-RA|32176;35357|32466;35376	311	207	212	1	0	0	1.2751	2.2751	2	6.9798
AAEL001913|AAEL001913-RA|794351|795389	1039	672	677	1	0	0	1.7765	2.7765	2	6.9747

Table 1. Top hits of potential miR-275 targets using a unique bioinformatic program based on Grimson et al. 2007. Highlighted is the top hit with 5 possible miR-275 binding sites, *d*Methyl Binding Domain R2 (*d*MBD-R2) or PHD Binding Domain.

Though a single bioinformatic analysis has been performed using the 'seed sequence' of miR-275, there is still much work left to be done in order to find its true gene targets. One method towards narrowing down the possible gene target candidates is using additional prediction programs based on different parameters. Though it has been suggested that one can use multiple prediction algorithms and compare them for overlapping hits, this method often produces

less than a 50% overlap and its use has been discouraged due to a lack of utility and rationale (Ritchie et al. 2009). One down side to using bioinformatic prediction is that most of them are based on sequence alignment alone. The many cases of imperfect base pairing between microRNAs to targets complicate the usefulness of prediction programs due to the possibility of important targets being left out (Wang et al. 2010).

Chapter 7. MBD-R2 Project

There is not much known about AAEL009416 (aka PHD finger domain or *d*MBD-R2) other than its conserved protein domains which predispose it as being linked to an activator or repressor of gene transcription and modification through histones and DNA methylation (Hendrich and Tweedie 2003). A previous study has already found *d*MBD-R2 to be linked to histone pre-mRNA processing machinery via RNAi knockdown in S2 cells (Wagner et al. 2007). Because the presence of a DNA binding domain is usually associated with transcription regulation, either through modification of DNA or transcription factors, it is possible that having unnatural levels of MBD-R2 can be causing down regulation of the PM genes.

In order to see if there is indeed a link between miR-275 and MBD-R2 a number of experiments were planned including expression analyses and RNAi knockdown. In collaboration with Dr. S. Aliyari, a researcher in Dr. Raikhel's

laboratory, primers and dsRNA against MBD-R2 were prepared. Using the tissues gathered at the beginning of the year, I first did a qRT-PCR expression analysis of MBD-R2 in wild type mosquitoes (Fig. 13).

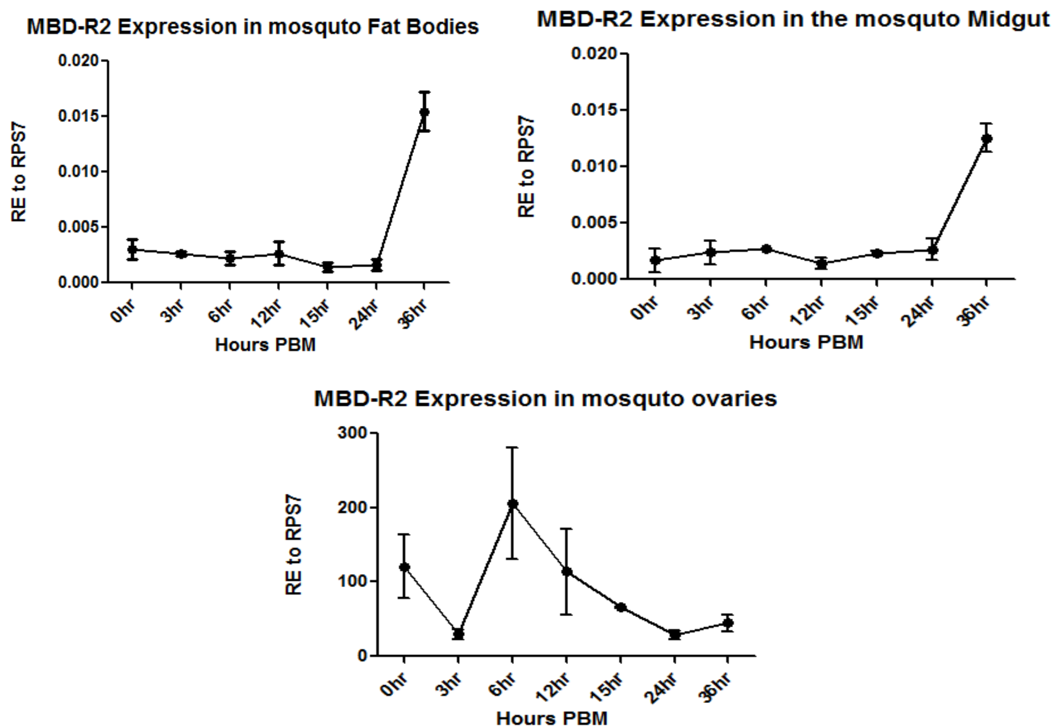

Fig. 13. Expression analyses of MBD-R2 in three different mosquito tissues. Tissues gathered from three different tissues (FB, MG, and OV) at different time intervals PBM were used for RNA extraction and qRT-PCR to observe expression patterns of potential miR-275 target, MBD-R2.

The expression analysis shows that there is an activation peak of MBD-R2 after 24 hours PBM in both FBs and MGs. The ovaries, on the other hand, are showing much higher expression levels and a different pattern that has a peak at 6 hours PBM. This great difference in the ovarian expression of MBD-R2 could be due to the multitude of genetic events occurring in developing embryos.

I then performed an RNAi knockdown of MBD-R2 by injecting dsRNA into the thoraxes of newly emerged female mosquitoes. Tissue was collected a few days later to check for knockdown efficiency and the remaining mosquitoes were blood fed by rat to see if there was any phenotype. It was thought there was a possibility that a phenotype similar to the knockdown of miR-275 might develop in the absence of its possible target, but that was not the case. There was no visible MG phenotype but there were some slight deficiencies in egg development (Fig. 14). In some ovaries select ovariols were smaller than others (Fig. 14 D, E), and in one case some ovariols seemed overdeveloped (Fig. 14 C). Knockdown was confirmed by qRT-PCR using mosquito FBs and MGs (Fig. 14 F). Though the same phenotype as knockdown of mir-275 was not obtained, this does not mean that MBD-R2 is not still a probable target. When miR-275 was knocked down, its targets were most likely up-regulated, not knocked down. So the miR-275 phenotype may be associated with an over expression of its target genes, meaning that a knockdown of its target will most likely not produce the same phenotype.

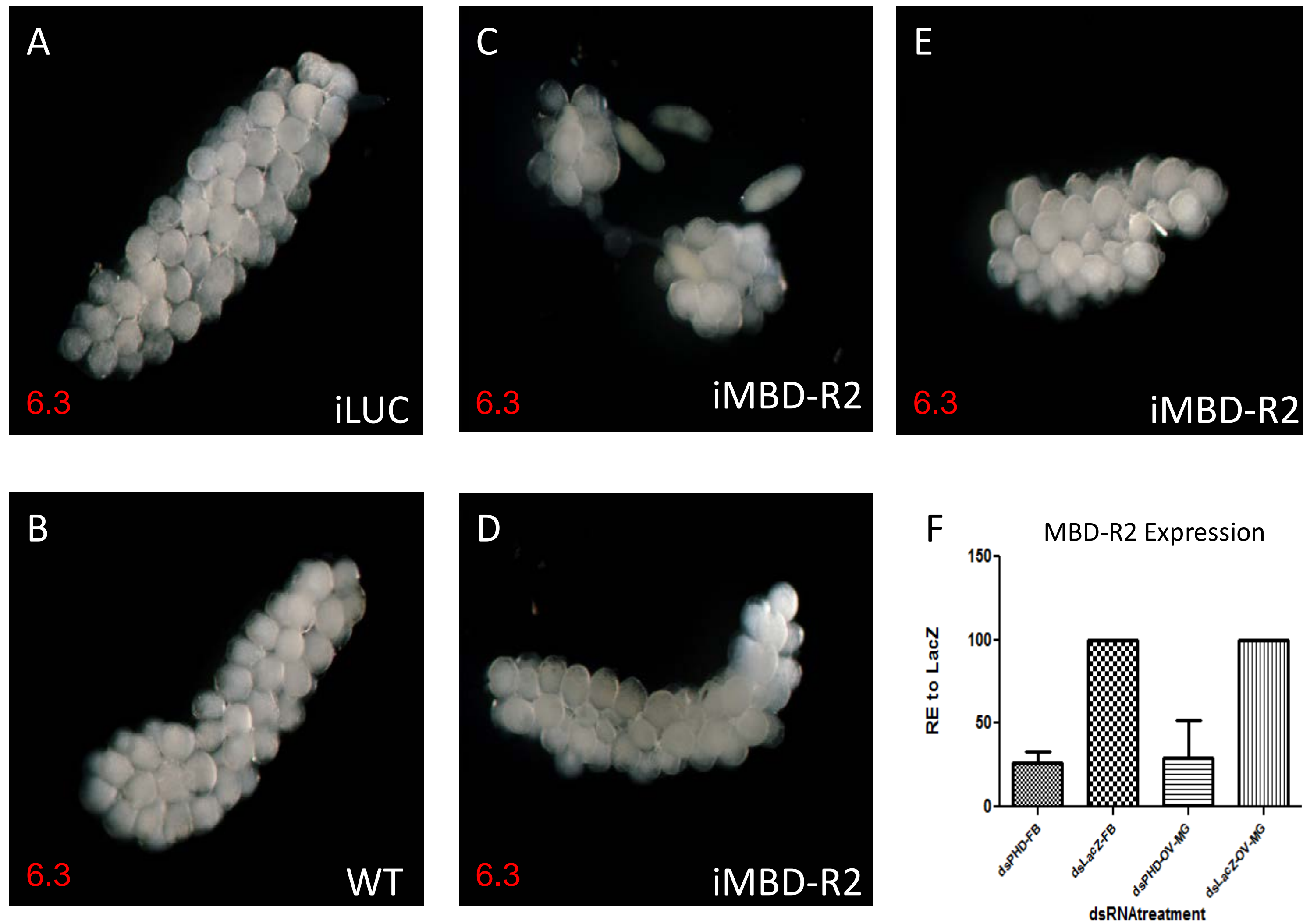

Fig. 14. RNAi knockdown of MBD-R2. (A,B) Ovaries of control mosquitoes (luciferace RNAi injected and wild type) 24 hour PBM. (C-E) Ovaries of MBD-R2 ds RNA injected mosquitoes 24 hours PBM showing some development deficiencies. (F) qRT-PCR showing knockdown efficiency of MBD-R2 (PHD finger domain).

Though the PM genes were not present on the bioinformatic prediction list, the effect of PM gene transcript knockdown on MBD-R2 expression was also tested (Fig. 15). Expression of both the MBD-R2 transcripts is lower in both PM gene knockdowns than iLacZ control, but it is on a fairly small scale.

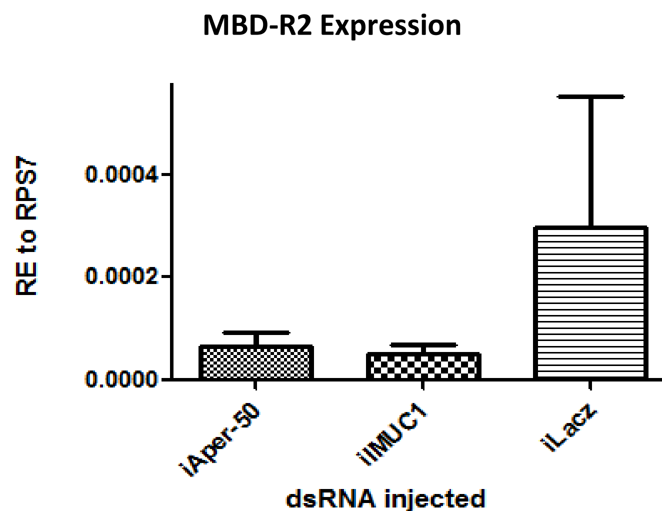

Fig. 15. Transcript levels of MBD-R2 are affected when peritrophic matrix (PM) proteins are knocked down. Levels of MBD-R2 transcripts appear lower the 24hrs PBM MGs of mosquitoes injected with dsRNA against PM proteins IMUC1 and APER50.

According to the mechanisms of miRNA regulation of gene expression, if a miRNA is knocked down or absent then its target gene(s) should be up-regulated because of the lack transcript repression. To test whether this were true for MBD-R2, I used the specific miRNA antagomir towards miR-275 to knock it down. I then harvested some of the Fat Bodies from positive phenotype mosquitoes to confirm knockdown and some were used in an expression analysis of MBD-R2. The qRT-PCR data in the graph (Fig. 16) shows that miR-

275 expression is down-regulated in knockdowns and MBD-R2 expression is significantly elevated (*P* value .030 vs WT, *P* value .017 vs MS) under the same conditions. This 2 fold induction of MBD-R2 during miR-275 knockdown makes a stronger case for MBD-R2 being a true target of miR-275.

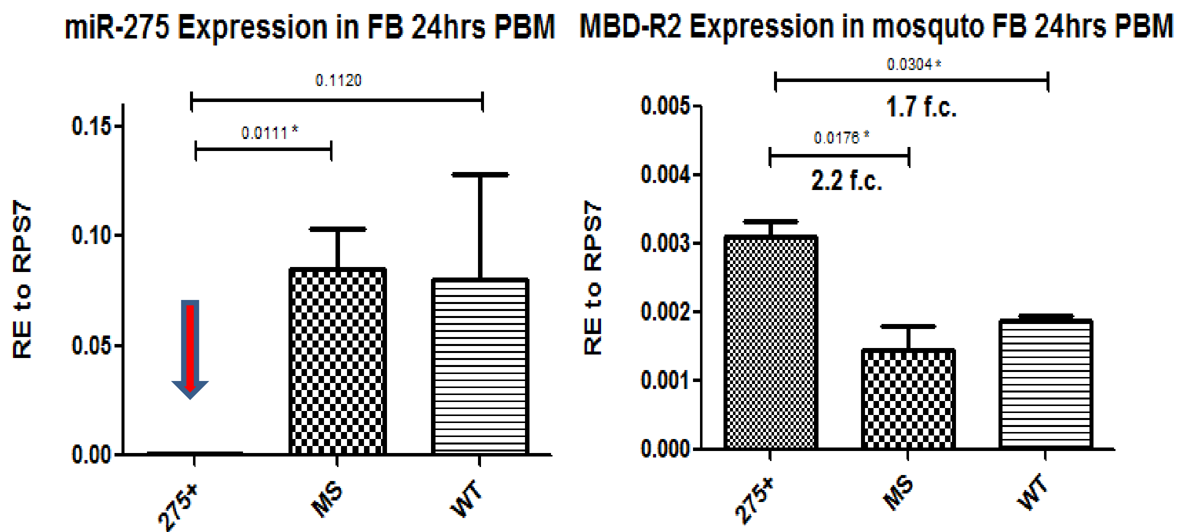

Fig. 16. MBD-R transcript levels are up-regulated when mir-275 is knocked down. The graph on the left shows confirmation of miR-275 knockdown by specific antagomir. The right shows up-regulation of MBD-R2 in FB taken from the same experiment.

Chapter 8. Conclusions and future directions

Results from the loss of miR-275 in *Ae. aegypti* show severe defects in ability to digest blood and develop eggs properly. What is still unknown is whether the disruption in egg development is only a downstream effect due to lack of proper nourishment received from blood or if two pathways are separately being affected. By fully exploring the specifics of the phenotype we were able to better understand what is being affected by the absence of this miRNA.

The integrity of the peritrophic matrix was show to be a factor contributing to irregular blood digestion phenotype associate with miR-275 knockdown. dsRNA knockdown of PM genes Aper50 and IMUC1 resulted in a visual phenotype similar to that of miR-275 knockdown. Though this does not tell us if the proteins of the PM are a direct target of miR-275, it does say that the PM is ultimately being affected. This theory is supported further by a preliminary Western blot showing that much of the Aper50 protein is absent in a positive phenotype miR-275-ant background. The phenomenon of blood excretion from the midgut should also be difficult with a proper peritrophic matrix in place because the PM forms a plug at the anterior portion of the midgut (Fig. 1). We believe that blood was able to escape the midgut and enter the crop sometime after blood feeding because no blood-in-crop phenotypes were ever observed immediately after a blood meal. An association between the digestion disrupting phenotype caused by miR-275 knockdown and two peritrophic matrix genes has

been established, however further understanding of this association would require more experimentation. The question still remains as to whether IMUC1 and APER50 are direct targets of miR-275. Finding out if would require specifically combing the 3'UTR of these two genes for potential binding sites and additional confirmation methods.

The discovery of a single miRNA affecting such important regulatory events as hematophagy and the gonotrophic cycle led to the question of its role in other damaging insect blood feeders. The preliminary work done on the malaria mosquito *Anopheles gambiae* has thus far shown that miR-275 serves the same purpose. The exact phenotype that miR-275 knockdown *Ae. aegypti* mosquitoes were afflicted with was visible in *An. gambiae*. Both blood meal regulation and ovary development were affected in about 25% of the 3 trials performed. Though many more trials and confirmations are needed, seeing that miR-275 and its apparent role are conserved between these very different mosquito species causes anticipation of how many other vector species it might affect.

Through use of a bioinformatics search target gene search one gene was identified as having 5 potential miR-275 binding sites in its 3' UTR. What little information that is known about Methyl Binding Domain-R2 (MBD-R2) is based on its conserved protein binding domains. It is thought to bind methylated DNA, but this raises the question of how it could fulfill that duty when mosquitoes have

very little methylated DNA in their entire genome (Marhold et al. 2004). It is still possible that it may regulate gene expression through these methods but more information about the gene is necessary before conclusions can be made.

While trying to shed light on the enigma of MBD-R2 even more questions were raised. The sharp up-regulation in MBD-R2 transcripts at 36 hours PBM in both FB and MG do not match up with the expression pattern of how a gene under the regulation of miR-275 should appear. This combined with the sporadic ovarial reaction to dsRNA knockdown of MBD-R2 only seem to raise more questions of the true role of MBD-R2 and if it is related to miR-275 in any way. Much more experimentation is needed to make conclusive remarks on if MBD-R2 affects the gonadotrophic cycle and if it is a direct target of miR-275. The addition of molecular based target search methods such as the immunoprecipitation of Ago and validation techniques to confirm binding are the next steps in determining the validity of MBD-R2 as a binding partner for miR-275.

The necessity of miR-275 in two major regulatory networks within mosquitoes is a critical discovery. Clues as to why it is so integrally important have been examined and they have so far lead in two directions: effects on the formation of the peritrophic matrix, and the *MBD-R2* gene. Though continued research into these two areas would yield some answers, more concrete evidence would come from using newer techniques to gain more insight into true miR-275 targets. As

these new potential targets are found, the real link between miR-275, the peritrophic matrix, and MBD-R2 can be ascertained.

Publication:

Bryant, B., Macdonald, W., Raikhel, A.S., microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito Aedes aegypti, *Proc Nat Sci USA,* 107 (52) 22381-22383 (2010).

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