Examining the roles of microRNAs in *Aedes aegypti* and *Drosophila melanogaster* spermatogenesis

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Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that act as regulators of gene expression at the post-transcriptional level in plants and animals. In animal cells, miRNAs typically bind with imperfect complementation to sequences within the 3′ UTRs of mRNAs, thereby inhibiting the translation of the transcripts. MiRNAs affect a variety of developmental pathways, and some of them appear to play important roles in defining the differential gene expression within the mammalian testis and during spermatogenesis; their functions in insects, however, remain largely unexplored.

In this study, I examined the expression of several putative testis-specific miRNAs in different tissues and developmental stages of the mosquito *Aedes aegypti* and the vinegar fly *Drosophila melanogaster*. MiRNAs -9, -34, -100, -124, and -219 were all expressed in the testes of the two insects, but some differences in their expression in other tissues were observed. One particular miRNA, miR-34, was examined more thoroughly, and was confirmed to target genes that either have functions in spermatogenesis or have a testis-specific expression pattern in the two dipteran insects. Inhibition of miR-34, using antisense oligonucleotides, and RNA interference-mediated knockdown of its target, *aae/014067*, in *A. aegypti* negatively impacted the fertility of the mosquito males. These results suggest that both *aae/014067* and miR-34 are clearly associated with *A. aegypti* male fertility and the disruption of their normal expression could render mosquitoes sterile, which would help in the development of sterile male release programs, to reduce the risk of mosquito-borne disease without the use of broad-spectrum pesticides that kill many non-target species.

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1. Introduction

Mosquitoes are the world's most serious disease vectors, transmitting diseases that kill millions of people each year (Iturb-Ormaetxe *et al*., 2011; McGraw and O'Neill, 2013). Efforts to reduce the transmission of the disease often focus on controlling pest mosquito populations close to human habitats using broad-spectrum pesticides (Ramirez *et al*., 2009; Raghavendra *et al*., 2011). As people become more concerned with the environmental impacts of current mosquito control methods, there is an increasing interest in finding alternative methods of controlling these serious pests. This study explores aspects of the molecular genetics of male mosquito reproductive biology in one of the most serious disease vectors, *Aedes aegypti*. The overall aim of the research is to identify new molecular targets for the future development of alternative control technologies that reduce our reliance on the existing pesticides. Before discussing these new approaches to mosquito control, it is worthwhile to first know more about the pest and how we have tried to reduce its disease-spreading potential.

1.1. The dengue vector *Aedes aegypti*

The mosquito *Aedes aegypti* is a serious vector of several important arbovirus diseases such as dengue fever, yellow fever, and chikungunya fever. According to the World Health Organization, dengue fever is the most important human arboviral disease, posing a major health problem in tropical and the subtropical regions (Rasheed *et al*., 2013). Dengue epidemics have been reported in over 100 countries, with approximately 100 million infections and at least 21,000 deaths worldwide every year (Arunachalam *et al*., 2008).

The yellow fever virus is endemic in tropical and subtropical areas of Africa and Latin America; with an estimated 200,000 people infected and 30,000 deaths worldwide every year (Mirzaian *et al*., 2010). Chikungunya disease is endemic to tropical Africa and Asia, but has had incursions into Europe and the Americas (Powers and Logue, 2007). An outbreak of chikungunya disease occurred in the islands of the Indian Ocean, affecting more than a million people in 2005-6, making this one of the biggest disease outbreaks in this region (Lahariya and Pradhan, 2006). Hundreds of thousands of cases of this disease are reported annually, and while patients suffer debilitating fevers and joint pain, relatively few people die from this disease (Lahariya and Pradhan, 2006).

Currently, there are few specific treatments or cures for most mosquito-borne diseases (World Health Organization). Although a vaccine has been developed for yellow fever, it remains a devastating disease in poor countries due to costly and limited mass vaccinations (Nene *et al*., 2007). Since these debilitating diseases are transmitted by the female of *A. aegypti*, finding effective control and management strategies for this species is a priority.

In addition to transmitting many important diseases, *A. aegypti* has other important negative impacts on humans. Since *A. aegypti* has a strong association with human habitation, living and breeding very near or inside human dwellings (Scott *et al*., 2000), these mosquitoes are a serious nuisance to people, hindering all kinds of outdoor recreation (Gubler, 2002; Rey *et al*., 2012). In addition, these mosquitoes have negative economic impacts, including reductions in real estate values, drops in tourism, and economic losses in agriculture and livestock (Gubler and Meltzer 1999; Gubler, 2002; Rey *et al*., 2012). As a consequence of both their disease vector potential and their

impacts on human activity and economics, mosquito control is a priority in developed areas where this species is found.

1.2. *Aedes aegypti* control

Currently, there are many methods used to control mosquitoes around the world, including surveillance, source reduction, the use of chemical pesticides, and biocontrol. Mosquito surveillance provides information on mosquito population size and species composition, as well as identifies breeding habitats and determines nuisance levels (Rey *et al*., 2012). Surveillance data, typically acquired using mosquito traps, are then used to guide mosquito control operations and to evaluate the effectiveness of control operations. Mosquito surveillance is most effective when combined with a program for monitoring environmental factors that may influence mosquito population change (Connelly and Carlson, 2009). However, mosquito surveillance can be costly and does not really provide a benefit to areas where mosquito-borne diseases are prevalent (Baly *et al*., 2007).

A. aegypti is closely associated with human environments, and makes use of almost any standing water source to lay eggs (Gubler, 1998, Toryo *et al*., 2008). Therefore, one way to control *A. aegypti* is to reduce water sources that are required for mosquito development. This technique can be as simple as properly discarding old containers, removing old tires or buckets, cleaning clogged gutters and changing water in bird baths (Gubler, 1989; Reiter and Gubler, 1997). Although control of *A. aegypti* populations through source reduction can reduce disease transmission, it has to be combined with others mosquito control techniques to be fully effective (Toryo *et al*., 2008).

Chemical pesticides that kill larvae (larvicides) or adult mosquitoes (adulticides) represent the most frequently used approach to control mosquitoes worldwide (McGraw and O'Neill, 2013). Control of *A. aegypti* larvae can be accomplished through use of chemical or biological agents and are applied directly to the water of mosquito breeding sites, where they prevent larvae from developing into adults. One of the most commonly used larvicides for *A. aegypti* control is temphos, which is an organophosphate that affects the central nervous system of the larvae (Lima *et al*., 2003; Da Silva-Alves *et al*., 2012). Chemical treatment for *A. aegypti* adults can be accomplished by ground and aerial applications (Lucia *et al*., 2008). Ground or residual treatment is applied in small areas to kill and exclude adults from resting sites often around the home (WHO, 2009). Spraying is typically in the form of thermal fogs or ULV (Ultra-Low Volume) aerosols, which are applied to large areas to kill adults when there is evidence that an epidemic is emerging (Bonds, 2012). Mosquito adulticides are considered the most effective approach for mosquito control due to rapid removal of adults in large areas, and are recommended for control in emergency situations such as during dengue epidemics (WHO, 2009).

While chemical insecticides have been shown to be effective in many contexts of mosquito control, their use in mosquito control programs has decreased in recent years, due to the high costs of synthetic insecticides and to growing concerns of the negative impacts on human health, non-target species, and evidence of increasing insecticide resistance in mosquito populations (Lima *et al*., 2003; Russel, 2004; Ghosh *et al*., 2012, McGraw and O'Neill, 2013). Resistance to a variety of commonly used chemical insecticides has been reported in *A. aegypti* populations around the world, due to

continued overuse of these insecticides in many localities (Lima *et al*., 2003; Marcombe *et al*., 2012; McGraw and O'Neill, 2013). For these reasons, alternative methods of mosquito control that are environmentally-friendly, cost effective, and target-specific are needed.

Biological control or biocontrol provides promising alternative approaches to manage mosquito populations that are environmentally-friendly, sustainable, and considered safer than conventional pesticides. These approaches are based on the use of natural enemies such as parasites, pathogens, predators, and botanical insecticides to target mosquitoes. Copepods have been used successfully in Vietnam to control *A. aegypti* larvae in water-storage containers where the mosquitoes breed (Kay and Vu, 2005; Sinh Nam *et al*., 2012). Another strategy uses *Wolbachia pipientis*, which is a parasitic bacterium that infects the reproductive system of insects and ultimately limits their ability to breed (Yen and Barr, 1973). *Wolbachia*-infected *A. aegypti* mosquitoes have been released in areas of Australia where outbreaks of dengue fever occur, leading to reduced lifespan of the mosquitoes, and thereby reducing their ability to transmit the disease (Iturb-Ormaetxe *et al*., 2011). A biological agent that serves as a larvicide in *A. aegypti* is *Bacillus thuringrensis israelensis* (Bti) (Mittal, 2003; Ritchie *et al*., 2010). Bti is a bacterium that contains protein crystals that rupture gut cells in mosquito larvae when ingested (Ritchie *et al*., 2010). Recently, botanical insecticides have been extracted from many plant families including Zingiberaceae, Bignoniaceae, and Solanaceae, and have potential to control *A. aegypti* populations (Choochote *et al*., 2005; Rodrigues *et al*., 2005; Raghavendra *et al*., 2009). Although biocontrol of mosquitoes has shown many

advantages over chemical insecticides, it is a slow process, can be costly, and can still affect some other non-target species.

Some research groups are considering genetic modification techniques to manage *A. aegypti* populations (reviewed in McGraw and O'Neill, 2013). Transgenic *A. aegypti* mosquitoes have been produced that contain RNA interference (RNAi) transgenes that can suppress dengue virus replication in the mosquitoes (Franz *et al*., 2006). Producing these insects within the lab is only a start; attempting to replace wild, dengue-competent mosquitoes with these transgenic mosquitoes will require the development of novel genetic drive mechanisms to ensure competitive fitness of the resistant mosquitoes (Wis de Valdes *et al*., 2011; McCraw and O'Neil, 2013). An older and more widely-used genetic modification method of controlling pest insects is the Sterile Insect Technique (SIT). SIT is based on the ability to mass-produce and release sterile males to compete for mates and thereby reduce a population. To sterilize the males, a variety of methods have been used, including exposure to gamma radiation, chemosterilization, cytoplasmic incompatibility or hybrid sterility (Nolan *et al*., 2011). If sufficient sterile males are released, they can effectively reduce the number of fertile matings, thus reducing the next generation's population. Repeated release of sterile males can reduce the population to low levels or for some insect pests, the population can be completely eliminated (Thome *et al*., 2010; Oliva *et al*., 2012).

SIT is a species-specific method of controlling insects that has negligible or no risk to humans or environment (Beech *et al*., 2009; Vasan 2010). The first successful use of SIT in the early 1950s involved the New World screw-worm, *Cochliomyia hominivorax*, a pest of livestock in the southern USA and later in Mexico, Central America, and

Northern Africa (Knipling, 1960; Lindquist *et al*., 1992). Later, SIT has been used to control a number of insect pest species, including the testse fly in Zanzibar (Vreysen *et al*., 2000), the Mediterranean fruit fly (medfly) within several continents (Hendrichs *et al*., 1995) and the apple codling moth in Canada (Bloem *et al*., 1999).

The tremendous success of SIT with many insect pest species suggested that SIT is a promising method for the control of mosquito populations. The first successful mosquito SIT trial involved the eradication of *Culex quinquefasciatus*, on an island off Florida between 1967 and 1974 (Dame *et al*., 2009). In this trial, releases of irradiated *C. quinquefasciatus* between 8,400 and 18,000 per day resulted in suppression and elimination of the populations in 10 weeks (Patterson *et al*., 1970). Another successful SIT program was used to control *Anopheles albimanus* in EI Salvador in 1971. Pupae were sterilized by chemosterilization solutions and around 40,000 sterile males were released daily at 14-15 km intervals, leading to complete elimination (Lofgren *et al*., 1974). Recently, sterile males of *Aedes albopictus* were released in northern Italy in a pilot trial, and the program is showing encouraging, although not immediate results (Bellini *et al*., 2007). The SIT approach is currently being used in trials in the Cayman Islands, Malaysia, and Brazil to control *A. aegypti* populations (Gubler, 2011).

Despite the early successes and the current trials of SIT for mosquitoes, the method still has its limitations (Benedic and Robinson, 2003; Alphey *et al*., 2010). Currently, radiation and chemosterilization are the primary methods that are used to produce sterile male insects; however, many trials have shown that radiation and chemosterilization can reduce competitiveness of sterile males, making them less able to compete with wild males (Dame *et al*., 2009; William *et al*., 2011; Oliva *et al*., 2012). Since SIT is based on

the distribution into the environment of large numbers of sterile males only (i.e. no females), mass rearing and sex separation are two objectives needed for optimal use of SIT to control mosquito populations (Dame *et al*., 2009; Alphey *et al*., 2010). Some important vectors, such as *Anopheles* species, have demonstrated considerable difficulties with both mass-rearing and sex separation techniques (Alphey *et al*., 2010).

One alternative to using radiation or chemicals for sterilization is to use genetic engineering to produce sterile insects. The availability of fully sequenced genomes for some mosquito species, coupled with transgenic technology, could potentially offer several advantages over the current approaches. For this approach to succeed, we need to identify genes involved in regulating male *A. aegypti* fertility, with the aim to disrupt their normal expression to render mosquitoes sterile.

1.3. Spermatogenesis – an introduction

Male fertility requires the production of large numbers of normal spermatozoa by the testis through a complex process known as spermatogenesis. A mammalian spermatozoon has organelles and structures in common with somatic cells, but often with a sperm-specific organization (White-Cooper, 2009). For example, the acrosome, which provides enzymes needed to penetrate an egg, is spermatozoa specific, while DNA packaging and chromatin remodeling in spermatogenic cells differ from packaging of DNA in other cell types. Similarly, the flagellar axoneme and the mitochondria in sperm have sperm specific components that differ from the axonemes and the mitochondria in other somatic cells. Therefore, spermatogenesis is a complex process involving many

different cell biological events to regulate sperm production in a cell type-specific manner.

In mammals, the male process of gametogenesis takes place in the seminiferous tubules of the testis (reviewed in Lie *et al*., 2010). The numbers of chromosomes that are present in diploid (2n) spermatogonia are halved when they develop into haploid (1n) spermatozoa, so that the genetic material can be restored to a diploid state following fusion with the egg. Spermatogenesis is comprised of several discrete steps, starting with mitosis for the renewal of spermatogonial cells, followed by meiosis I to produce primary spermatocytes, which then enter meiosis II to form haploid round spermatids. Spermatids then undergo a process known as spermiogenesis, during which the genetic material is highly condensed in the nucleus of the spermatid head. The acrosome forms above the head, and the spermatid tail elongates. Lastly, spermiation occurs, during which fully developed spermatids detach from the seminiferous epithelium, enter the tubule lumen, and are transported to the epididymis for maturation.

1.3.1. Spermatogenesis in the model insect *Drosophila melanogaster*

Much of what we know about the molecular genetic controls of spermatogenesis has been derived from using model species to identify key genes and developmental pathways. The vinegar fly, *Drosophila melanogaster*, has been heavily used in genetic research and is a common model organism for the study of developmental and cell biological processes. This insect has a number of features that makes it an ideal species for study, including a short generation time, a high fecundity, a relative ease of genetic manipulation, an availability of genetic resources, and a fully sequenced genome. The

stages of *Drosophila* spermatogenesis are well defined, the cells are large and easily accessible, and there is evidence that many aspects of the genetic control of spermatogenesis are conserved throughout many taxa (White-Cooper, 2009).

The imaginal discs of *Drosophila* are groups of primordial cells that sit aside during embryogenesis, proliferate during the larval period and differentiate during metamorphosis. They differentiate into adult epidermal structures, such as the eye, antenna, leg, wing and terminalia (reviewed in Estrada *et al*., 2003). Early in embryogenesis, three clusters of primordial cells that are found in embryonic abdominal segments fuse and form the genital disc which gives rise to the adult terminalia, including genitalia and analia (Sanchez and Guerrero, 2001). Cells in the genital disc proliferate during larval stages and by the third larval stage, male and female genital discs exhibit clear morphological differences (Estrada *et al*., 2003). The male genital disc gives rise to both the external and internal genitalia (except gonads), such as the genital arch, the claspers, the penis apparatus and the vas deferens (Chen and Baker, 1997).

In *Drosophila*, the embryonic gonad is composed of two primary cell types, primordial germ cells (PGCs) and mesodermally-derived somatic gonadal precursor cells (SGPs) (Okegbe and DiNardo, 2011). The PGCs develop at the posterior end of the embryo, and they migrate through the endoderm to reach the mesoderm (Jemc, 2011). While the PGCs are migrating, the SGPs are specified from the lateral mesoderm and begin integration with the PGCs at stage 11 (6 hours after fertilization) (Okegbe and DiNardo, 2011). The PGCs and the SGPs then migrate together anteriorly resulting in a rounded, compacted gonad by stage 15 (13 hours after fertilization) (Jemc, 2011). Between stages 15-17 of embryonic development, the gonad exhibits sexually dimorphic

differences, as male-specific cells appear, including male-specific somatic gonadal precursor cells (msSGPs) and pigment cells (DeFlaco *et al*., 2008).

The adult *D. melanogaster* testis is a blind-ended tube comprised of muscle and pigment cells on the exterior and male germline and somatic support cells on the interior. A specialized cluster of post-mitotic somatic cells known as the hub at the distal end of the testis is the site spermatogenesis. Hub cells become specified late in embryogenesis, as they are not visible until near hatching of the first larval instar. The hub is surrounded by male germline stem cells (GSC), which are derived from the PGCs located at the anterior of the gonad, and somatic stem cells known as the cyst stem cells (CySCs). Each mitotic division of germline stem cells regenerates a GSC, and produces a spermatogonium. Similarly, each mitotic division of somatic cyst cells regenerates a cyst stem cell and usually produces a cyst cell. The two cyst cells encapsulate each spermatogonium and proceeds through four mitotic divisions to generate 16 primary spermatocytes. The primary spermatocytes progress through meiotic divisions to end up with 64 round spermatids (reviewed in Fuller, 1993; Jenkins *et al*., 2003; Le Bras and Van Doren, 2006).

The round spermatids undergo dramatic morphological changes to transform themselves into elongated cells. As the 64 spermatids from each single spermatogonium are still encysted, and interconnected, they polarize in concert. During spermatid elongation, differences between the two cyst cells become apparent. The head cyst cell covers the caudal end of the spermatids, while the tail cyst cell encapsulates the elongating tails. The cyst orients with the spermatid heads pointing toward the base of the testis, while the tails elongate towards the apical testis tip. Finally, the fully elongated

spermatids undergo individualization. Sperm individualization generates mature sperm which coil at the base of the testis before release into the seminal vesicle (reviewed in White-Cooper, 2010). Thus, male fertility depends on several developmental and physiological processes through gonad development and spermatogenesis. Defects at any process can result in a failure to continue producing functional gametes and a subsequent reduction or loss in fertility.

1.3.2. Spermatogenesis in mosquitoes

The basic processes of spermatogenesis are similar in very different animals, and the genes responsible are highly conserved (Bonilla and Xu, 2008). In mosquitoes, spermatogenesis occurs mainly during the larval and pupal stages of mosquitoes, but different processes can occur during these developmental periods and can vary in different species. For example, there are three main stages of testes growth in *Aedes stimulans*. The germ cells become globular and the testes increase in the length during the first instar period. During the second instar and the first half of the third instar, the testes doubles in length and the germ cells become enclosed in cysts. Spermatozoa appear through most of the testis 95 hours after larval-pupal ecdysis, and at the end of pupal stage, sperm cells lie free within the testis cavity and the most proximal part of the vas efferens. Spermatogenesis may continue during the adult stage but with a decreasing rate. In *Aedes aegypti*, new cells are formed after the male has become sexually mature, and some spermatogenesis continues for more than 10 days, but the rate of sperm production is significantly reduced relative to the immature adult. However, spermatogenesis in other mosquito species stops completely by the time the male is capable of copulation (reviewed in Clements, 1992).

1.3.3. Genetic controls of spermatogenesis

Genomic studies in *Drosophila melanogaster* have revealed that testis-specific genes are underrepresented on the X chromosome (Vibranovski *et al*., 2009). Several studies have noted that the testis-specific genes have been copied and moved from the X chromosome to the autosomes and that the autosomal copy is much more likely to have testis-specific expression than the X-linked copy (Meiklejohn *et al*., 2003; Parisi *et al*., 2003; Ranz *et al*., 2003). The inactivation of X-linked genes during male meiosis may explain the accumulation of testis-expressed genes in autosomes (Vibranovski *et al*., 2009). Nevertheless, there are many genes that are testis-specific or testis -enriched on the X chromosome. For example, 13 out of 50 testes -enriched genes are located on the *Drosophila* X chromosome, including genes of the *Sdic* and *tektin* gene families (Ranz *et al*., 2003; Hense *et al*., 2007).

Several gene pathways have been identified in the germinal proliferation center at the apical region of the *Drosophila* testis such as the JAK-STAT pathway and the Dpp pathway (Tulina and Matunis, 2001; Kawase *et al*., 2004). The JAK-STAT pathway is essential for stem cell-renewal in the *Drosophila* male germ line. *Drosophila* males with mutations in the JAK pathway lacked a renewing germ line, which suggests that JAK plays a key role in the maintenance of germ line stem cells. The JAK-STAT pathway needs a signal from surrounding cells to be activated. Unpaired (Upd) has been identified as a factor required to activate the JAK-STAT pathway in the *Drosophila* testis (Tulina and Matunis, 2001), but additional, yet to be identified, factors likely assist in renewing the germ line (Kiger *et al*, 2001). Microarray profiling in various adult tissues of *Drosophila* shows that about 50% of the genes in the genome are expressed in testes,

with 8% of the transcripts detected in adults being testis-specific, and 5% being testis-enriched. In contrast, 1.5% transcripts were brain-specific and only 0.5% transcripts were detected exclusively in ovaries (Chintapalli *et al*., 2007). Most of these testis-specific genes or testis-enriched genes are expressed in the primary spermatocytes stages, and continue into spermatid elongation stages and later in spermiogenesis (White-Cooper, 2010). Many genes have been identified in *Drosophila* primary spermatocytes as testis-specific genes such as, *βtub85D* (*β2t*), *fzo*, *aly*, *Porin2*, and *boule* (Dorus *et al*., 2006; Chintapalli *et al*., 2007). For example (*β2t*) is responsible for the meiotic spindle and axoneme structural components (Kemphuse *et al*., 1979). However, most of the testis-specific gene functions remain uncharacterized (White-Cooper, 2010).

1.4. MicroRNAs as gene regulators

MicroRNAs (miRNAs) are single-stranded, noncoding small RNAs of approximately 22 nucleotides. In animal cells, they act as post-transcriptional regulators that usually bind to complementary sequences on the 3` untranslated regions (UTRs) of target messenger RNAs, which can result in gene silencing via translational repression or target degradation. MiRNAs are endogenous molecules; they are either expressed from independent transcriptional units or derived from introns of protein-coding genes or introns of long non-coding RNAs (Rodriguez *et al*., 2004; Griffiths, 2007). The first miRNA genes, *lin-4* and *let-7*, were discovered in *Caenorhabditis elegans* (*C. elegans*), and shown to be involved in timing of early development of the worm (Lee *et al*., 1993). Since their discovery in the 1990s, hundreds of miRNAs have been identified in various eukaryotic organisms including plants, primates, birds, fish, worms, and flies, and recently, they have been identified in viruses and in the unicellular algae *Chlamydomonas*

reinhardtii (reviewed in Papaioannou *et al*., 2010). MiRNAs are highly conserved across species and are involved in the regulation of different cellular processes such as developmental timing, cell differentiation, cell proliferation, apoptosis and metabolism (Chen, 2004; Brennecke *et al*., 2003; Xu *et al*., 2003; Bushati and Cohen 2007; Ambros, 2011). Although a plethora of miRNAs are found in many species, the function of the vast majority of them has not been identified yet (Ying *et al*., 2012).

1.4.1. Biogenesis of microRNAs

Most miRNA genes are transcribed by RNA polymerase II (Pol II), but a small group of miRNAs can be transcribed by polymerase III (Faller *et al*., 2008). Pol II transcribes a miRNA gene to produce the primary miRNA (pri-miRNA) (Figure 1.1). Pol II- derived pri-miRNAs are 5' capped, spliced, and polyadenylated (Cai *et al.,* 2004). In the nucleus, the pri-miRNA is processed by a multi-protein complex called Microprocessor, which cleaves the pri-miRNA into a shorter hairpin-structured precursor (pre-miRNA) (Siom *et al.,* 2010). Microprocessor consists of the double-stranded RNA binding protein called DGCR or Pasha in invertebrates, and Drosha, which is an RNase III enzyme. DGCR8/Pasha binds to the junction between the single-stranded and double-stranded regions of the pri-miRNA stem and directs Drosha to cleave 11 bp away from the junction, resulting in a molecule of about 70 nucleotides long, with a two-nucleotide overhang at the 3`end.

The pre-miRNAs are exported from the nucleus by exportin 5 (EXP5), which is a member of the nuclear transport receptor family and required for nuclear export of both short hairpin RNAs (shRNAs) and pre-miRNAs (Yi *et al.,* 2005). This protein recognizes

the two –nucleotide overhang left by Drosha and transports the pre-miRNAs into the cytoplasm by a Ran-GTP-dependent mechanism (Okada *et al.,* 2009). In the cytoplasm, pre-miRNAs are cleaved by the RNaseIII enzyme Dicer, producing a 22-nt miRNA duplex (Kim *et al.*, 2009). After Dicer cleavage, the duplex is separated by the RNA-induced silencing complex (RISC), which contains the Ago protein (Siom *et al.*, 2010). One 22 nt strand remains bound to the Ago protein as the mature miRNA (the guide strand), whereas the other strand (the passenger strand or miRNA*) is degraded. The thermodynamic stability at the two ends of the miRNA duplex determines which strand is the guide strand (Khvorova *et al.*, 2003). The miRNA strand with the relatively unstable base pairs at the 5` end will be more frequently chosen as the guide, while the miRNA strand with relatively stable base pairs at the 5` end will be degraded. However, recent studies show that either the miRNA or miRNA* strands can be functional; in this case, the miRNA* strand is not degraded, but associates with Ago protein (Okamura *et al.*, 2009).

Typically, animal miRNAs bind to the 3`UTR region of the target mRNA with imperfect complementarity, with most miRNAs having a seed region ( nucleotides 2-8 at 5`end) that are required to trigger miRNA-mRNA interaction and result in translational repression, cleavage, or destabilization (Zhang *et al.,* 2009). However, there is also evidence that mammalian miRNAs can bind to coding regions or even to 5`UTR sites of target mRNAs (Lytle *et al*., 2007; Schnall *et al*., 2010). Due to imperfect binding between a miRNA and mRNA in animal cells, one miRNA can target many different sites on the same mRNA or many different mRNAs, so a single miRNA can regulate different genes at the post-transcriptional level (Zhang *et al.*, 2009).

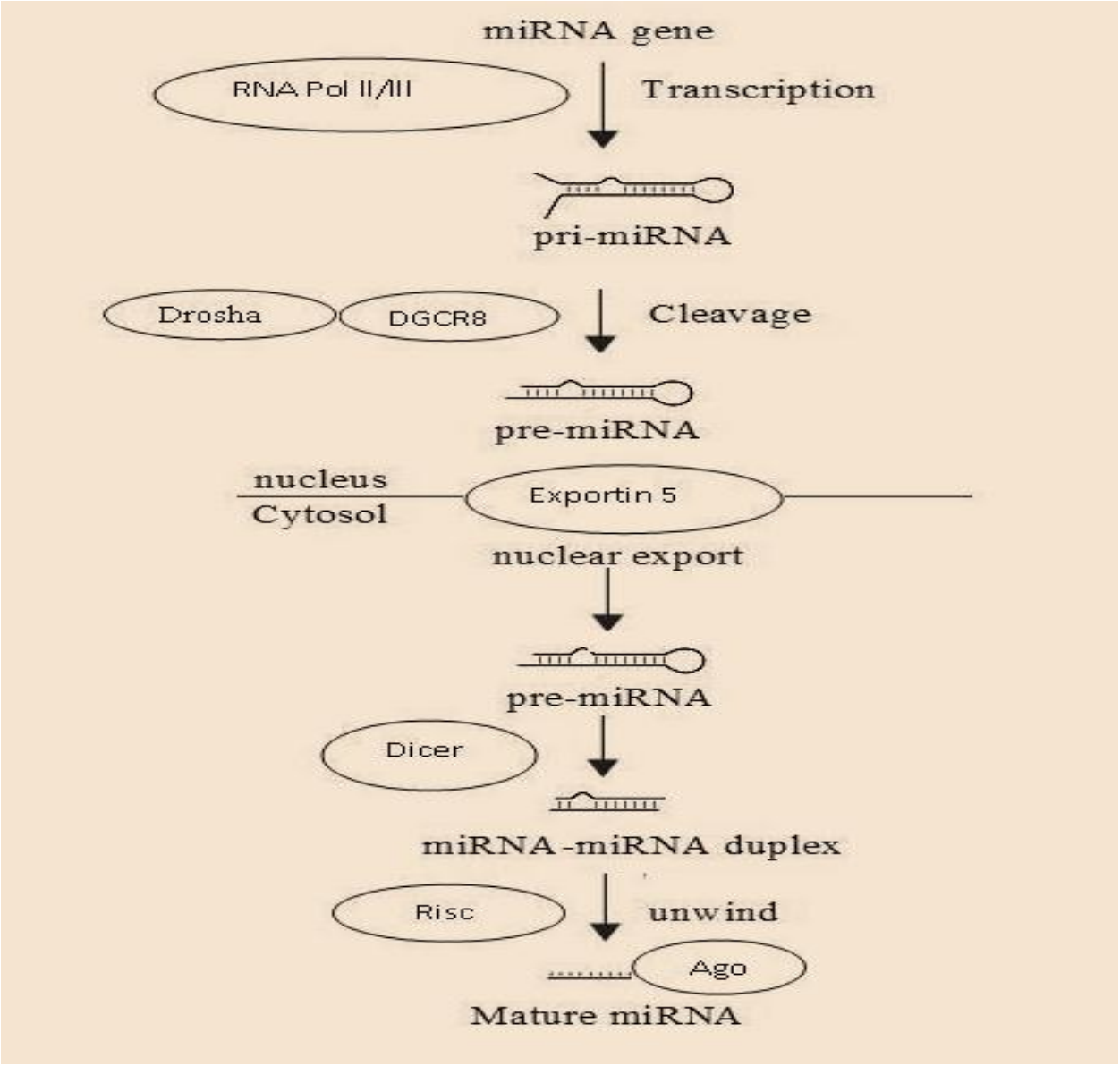


Figure1.1. The microRNA biogenesis pathway.

1.4.2. Plant microRNAs

While core components of the miRNA pathway are conserved between animals and plants, many aspects of miRNA biogenesis and function are different in the two kingdoms. For example, the entire process of miRNA biogenesis in plants is undertaken within the nucleus in specialized subnuclear regions termed D-bodies. The mature miRNAs are transported out of nucleus by Hasty, an exportin 5-like protein found in plants (Park *et al*., 2005; Song *et al*., 2007). Furthermore, plants miRNAs usually have perfect or near perfect pairing with their mRNA targets and usually induce gene repression through direct cleavage of their target transcripts (Rhoades *et al*., 2002). That could explain why all known plant miRNAs have single target sites on their target mRNAs and regulate the expression of just one gene (Axtell *et al*., 2011). Unlike animals, plant miRNAs may bind to their targets in 5`UTRs, ORFs and 3`UTRs, as well as within non-protein coding transcripts (Axtell *et al*., 2011).

1.4.3. MicroRNAs as regulators of spermatogenesis

Post transcriptional regulation has emerged as a major process that regulates the protein synthesis in developing male gametes (He *et al.,* 2009). The major cellular changes in spermatogenic cells are strongly dependent on post- transcriptional processes because transcription ceases before the completion of spermatogenesis (reviewed in Braun, 1998). Recently, miRNAs have been identified as regulators of gene expression in mammalian spermatogenesis; in particular they regulate genes in germ cells that regulate the complex process of stem cell renewal and/or differentiation (He *et al.,* 2009).

Many expression-profiling studies have observed that the chromatoid bodies in meiotic and early haploid male germ cells express a number of miRNAs in the mammalian testis, suggesting that miRNAs may be act as important regulators of spermatogenesis (Kotaja *et al.*, 2006; Ro *et al.*, 2007). Some of these miRNAs are testis-specific, such as miR-t17 and miR-t25, but most of them are testis-preferential (Ro *et al.*, 2007). For example, miR-122a is predominantly expressed in late stage male germ cells, and it represses the transition protein (Tnp2) which is necessary for chromatin condensation during mouse spermatogenesis (Yu *et al.*, 2005). MiR-34b is expressed much more in the adult mouse testis compared with a prepubetal testis, which indicates that miR-34b may be play a role in the differentiation of mouse male germ cells (Barad *et al.*, 2004). However, the functions of most miRNAs that have been identified in the mammalian testis are still unknown (He *et al.*, 2009). Similarly, miRNAs have been identified in testes of insects, but the function of most of these miRNAs have not been determined (Aravin *et al*, 2003; Skalsky *et al*, 2010). The only testis miRNA in *Drosophila* that has had a function ascribed is miR-7, which appears to play a role in GSC maintenance by repression of *bam* mRNA (Cuevas *et al.*, 2011). Many of the miRNAs found in *D. melanogaster* appear to have homologues in mosquitoes, but their functions have not been ascribed either (Behura *et al.*, 2006).

1.4.4. Computational tools for microRNA target prediction.

Several computational algorithms have been developed to predict mRNAs targeted by miRNAs. These algorithms are mainly focused on sequence alignments to identify complementary elements between the seed region at the 5`-end of the miRNA and the 3`UTR of the mRNA (reviewed in Yeu *et al.*, 2009). Most algorithms also use additional

steps to refine the predictions and rank them according to statistical confidence (Ritchie *et al*., 2013). miRanda, MicroInspector, and PITA algorithms for example, calculate the thermodynamic stability of miRNA:mRNA duplexes by searching for the strongest physical interactions between the seed region at the 5`-end of a miRNA and the 3`UTR of putative mRNAs (John *et al*., 2004; Rusinov *et al*., 2005; Kertesz *et al*., 2007). However, this approach is limited by accurate prediction of stable secondary structures (Ritchie *et al*., 2013). Another approach that is used for prediction of miRNA targets involves evaluating sequence conservation of predicted targets between different species. For example, TargetScan predicts biological targets of miRNAs by searching for the presence of conserved sites that match the seed region of a miRNA among different vertebrate species (Lewis *et al*., 2005). This approach reduces the number of false positive predictions, but it has little use in detecting species-specific binding sites (Ritchie *et al*., 2013).

While computational algorithms have been the major tool for prediction of miRNA targets, all of the miRNA:mRNA interactions examined by these algorithms are merely predictions and have not been functionally validated. In addition, most prediction algorithms are restricted to examining the 3`UTR of the mRNA and do not incorporate evidence of functional binding between the miRNA and the 5`UTR or protein coding region of the mRNA (Thomson *et al*., 2011). Moreover, the stable pairing between miRNA and 3`UTR of the mRNA may not necessarily be a functional interaction, which may explain why the false positive rate of prediction by these algorithms is still high (Kuhn *et al*., 2009; Thomson *et al*., 2011). Therefore, experimentation is essential to identify genuine miRNA targets.

1.5. Thesis Research Objectives

The broad aims of this research project are to identify some miRNAs associated with testis development and/or spermatogenesis in the mosquito *Aedes aegypti*, and to determine whether these miRNAs share conserved functions in the vinegar fly *Drosophila melanogaster*. Identification of new molecular targets in male mosquito reproductive biology will provide some possible new directions for future development of alternative control technologies that reduce our reliance on the existing pesticides.

The genomes of both insects have been sequenced, and putative homologues of the *D. melanogaster* miRNAs can be identified in the genome of *A. aegypti* using BLAST analyses. These *in silica* analyses do not however, provide any information on the functional roles of the miRNAs in either species, and in this study, I will focus on assessing the functional role of a small subset of miRNAs, with particular emphasis on identifying some of the miRNAs and their targets that are associated with testis development and/or spermatogenesis.

The specific objectives are described below:

Identify a subset of microRNAs found in both *D. melanogaster* and *A. aegypti* that could have a role in regulating genes involved in spermatogenesis or testis development.

Many miRNAs are found to be conserved among different organisms, and they regulate biological functions common between invertebrates and vertebrates (Lee *et al*., 2007; Ibáñez-Ventoso *et al*., 2008). Using bioinformatics tools, several putative testis-related miRNAs were identified in the genomes of *Aedes aegypti* and *Drosophila melanogaster,* based on miRNA homologues found in humans and mice that are

expressed either exclusively or primarily in the testis (Yan *et al.*, 2009; Buchold *et al*., 2010; Dyce *et al*., 2010; Niu *et al*., 2011).

Identify a subset of target genes for candidate microRNAs in both *D. melanogaster* and *A. aegypti*.

Using computer algorithms, predicted target genes of the miRNAs in *Aedes aegypti* and *Drosophila melanogaster* were examined, with the aim to identify those genes with known testis expression in other species.

Experimentally determine the tissue-specific, sex-specific and developmental stage-specific expressions of the identified microRNAs.

Many miRNAs in animals exhibit tissue-specific or developmental stage-specific expression (Lagos-Quintana *et al*., 2001; Lee *et al*., 2001). qRT-PCR was used to assess whether each of the identified miRNAs expression was restricted to the testis or may also be expressed in other tissues.

Assess the functional roles of some microRNAs by testing their ability to bind to the 3`UTR sequences of predicted target genes.

MiRNA target prediction programs only suggest which genes are regulated by a miRNA, but do not provide confirmed functions of the miRNAs (Kuhn *et al*., 2009). Therefore, a cell-based functional assay for the identification of miRNA targets was used in this study to assist in determining the true binding potential of miRNAs to their target mRNAs. This assay is based on the binding of a given miRNA to its specific mRNA target site, thereby repressing expression of a GFP reporter gene that is linked to 3` UTR of mRNA.

Assess whether perturbations in a microRNA's and a target gene's expression affect male fertility.

A loss-of-function technique is a powerful method for determining the role of a specific gene. Knock-down of the expression of either a testis-microRNA itself or of one of its target genes could impact the fertility of the mosquito and ultimately could render mosquitoes sterile. Antisense oligonucleotides and dsRNA were injected to mosquitoes to investigate the role of miRNA and its target, respectively, with respect to male fertility.

2. Methods

2.1. Insects Rearing

A *Drosophila melanogaster* white-eyed strain (w1118) was used for all experiments. Stocks were maintained at room temperature on a potato flake medium (Ward's Instant Drosophila Medium).

McAllen strain *Aedes aegypti* were reared at 25ºC, 50% humidity, on a 12h light: 12h dark photoperiod. Eggs were placed in small tubs of dechlorinated tap water with ground desiccated liver tablets to induce hatching. Larvae were maintained at 27ºC in these tubs until pupation. Pupae were then collected and placed in large cages for eclosion, and adults were maintained on 10% sucrose solution. Females were blood fed once a week by creating a blood bag from stretched Nescofilm (Karlan Research Products) containing ~2 ml of blood obtained from rats from the Animal Holding Faculty at the University, Fort Garry Campus. Blood was warmed to 42Cº prior to feeding, and females were allowed to lay eggs on dampened paper towels. Laid eggs were dried and stored in humid containers prior to hatching.

2.2. Identification of candidate microRNAs

A subset of miRNAs have been identified in humans and mice that are expressed either exclusively or primarily in the testis (Yan *et al.*, 2009; Buchold *et al*., 2010; Dyce *et al*., 2010; Niu *et al*., 2011). By using MiRBase (http://www.mirbase.org/) to investigate the general features of candidate miRNAs described in the literature, putative homologues of these miRNAs in the genomes of *Aedes aegypti* and *Drosophila melanogaster* were identified.

2.3. Identification of potential microRNA gene targets

Two databases were used to determine predicted targets of the identified miRNAs. The databases used for this investigation were TargetScanFly (http://www.targetscan.org/fly_12/) and MicroInspector software (http://bioinfo1.uni-plovdiv.bg/cgi-bin/microinspector/). Each database uses its own algorithm to predict miRNA:mRNA 3'UTR binding based on various parameters, including the presence of conserved 8mer and 7mer sites that match the seed region of the miRNA and the free energy of binding the miRNA to its predicted target (Ritchie *et al.* 2013). Top predicted targets were compared between the databases and genes involved in testis or spermatogenesis functions were identified using FlyBase (http://flybase.org/) gene annotations. Approximately 500 bp of sequence immediately downstream of the last exon, representing the putative 3`UTR, was obtained from FlyBase (http://flybase.org/) and VectorBase (https://www.vectorbase.org/).

2.4. Determining tissue-specific and sex-specific expression of the putative microRNAs

RNA was extracted from dissected tissues of approximately 60 adult male and 60 adult female five-day-old *A. aegypti* mosquitoes. The tissues included testes, ovaries, midguts, and the remaining carcasses lacking the aforementioned tissues. Tissue was dissected in phosphate buffered saline (1 X PBS, pH 7.4; Sigma) using ethanol-treated forceps and immediately placed in1.5 ml microfuge tubes containing 600 µl RLT lysis buffer and 1% ß-mercaptoethanol for homogenization. RNA was then extracted using a Qiagen RNeasy RNA kit according to the manufacturer's instructions. Firstly, the

dissected tissues were crushed using a plastic pestle in lysis buffer. The mixture was then placed in a Qiagen QiaShredder column for tissue homogenization and the resulting lysate was used with RNeasy extraction kit. Extracted RNA was eluted in 50 µl of RNase –free water and concentrations were determined using a NanoVue (GE Healthcare) spectrophotometer. RNA from *D. melanogaster* dissected tissues (testis, ovaries, and remaining carcasses lacking testis and ovaries), extracted as described above, which was kindly provided by Natalie Doughty.

For cDNA synthesis, RNA (0.8-1 µg) was treated with gDNA Wipeout Buffer (Qiagen) to remove any contaminating genomic DNA from the samples. RNA was reverse transcribed to synthesize cDNA using a Quantitect Reverse Transcription kit (Qiagen) with oligo-dT and random primers according to the manufacturer's instructions. Samples were incubated with Quantiscript Reverse Transcriptase (Qiagen) for 15 min and the reaction was terminated by incubating at 95ºC for 3 min. The concentration was determined using a NanoVue (GE Healthcare) spectrophotometer.

To determine the relative expression levels of the candidate miRNAs (miR-9, miR-34, miR-100, miR-124, and miR-219) in different tissues of *A. aegypti* and *D. melanogaster*, quantitative reverse-transcriptase PCR (qRT-PCR) was performed using a BioRad iQ5 Real-time PCR detection system. For each cDNA sample, qRT-PCR was performed in triplicate using a 96-well plates with 20 µl reactions containing ~10 ng of cDNA, 10 µl of SYBR Green Supermix (BioRad), 1 µl each of forward and reverse primers (10 µM), and Nanopure water, using the following program: 95ºC for 3 min, followed by 40 cycles of 95ºC for 10 sec, then the annealing temperature for 30 sec, followed by a melt curve analysis to confirm that only a single PCR product was

amplified. qRT-PCR primer sets were designed using Primer3 Input (version 0.4.0) (http://primer3.wi.mit.edu/), to amplify the ~70 nt pre-microRNA sequences. Primers then were verified using Sigma DNACalc (http://www.sigma-genosys.com/calc/DNACalc.asp). Primer sequences are listed in (Table 2.1). Species-specific ribosomal *S7* primers (Fwd: 5`AATAAATTCGCTATGGTTTTCGG and Rev: 5` CCTTCTTGCTGTTGAACTCG) in *A. aegypti* as well as ribosomal *Rpl32* (Fwd: 5`AAGGGACAGTATCTGATGC and Rev: 5`CACCAGGAACTTCTTGAATC) in *D. melanogaster* were used as an internal reference to compare relative levels of gene expression.

Table 2.1. QRT-PCR primers used to detect target gene fragments from *A. aegypti* and *D. melanogaster* cDNAs. (F = forward primer, R = reverse primer)

Species	miRNA	Sequence(5`-3`)	Product Size
A. aegypti	miR-9a	F:GTCAAAGTTCTCTTTGGTTATCTAGC R:ATTAACTTCGGTATGCTAGCTTTATG	74bp
D. melanogaster	miR-9a	F: TATGTTGTCTTTGGTTATCTAGCTG R:AGCTAGCTTTATGACGTTATTTATCA	57bp
A. aegypti	miR-34	F: ATACGCTATGGCAGTGTGGT R: GCGGATAGTGGTTGTGAATC	101bp
D. melanogaster	miR-34	F: ATTGGCTATGCGCTTTGG R: GCGGCAGTGAAGATAGTGG	88bp
A. aegypti	miR-100	F: GCGTAGTGGTTGTAGTTACCCA R: CGATCGATGCTTTAGCCATA	120bp
D. melanogaster	miR-100	F: AACCCGTAAATCCGAACTTG R: TTGCATTGACAGACTCCCATA	72bp
A. aegypti	miR-124	F: TGCACGTTTTTCTCCTGGTA R: AATTGCGTTCGCTCTTGG	92bp
D. melanogaster	miR-124	F: TTTGGTACGTTTTTCTCCTGGT R: TAGAACTGCGTTCGCTCTTG	92bp
A. aegypti	miR-219	F: TCTAGCTCTGATTGTCCAAACG R: AGCCACGGATGTCCAGTC	79bp
D. melanogaster	miR-219	F: GCTATGATTGTCCAAACGCAAT R: TTATTTCGAGCCGCGATG	77bp

Cycling conditions and dissociation curve analysis were performed on the iQ5 Thermal Cycler (BioRad), according to the manufacturer's instructions. The relative amount of microRNAs in *A. aegypti* and *D. melanogaster* was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where gene transcript levels are normalized to the internal standard (*S7rp*) and (*Rpl32rp*) using the following equation: $\Delta\Delta CT = (CT_{,miRNAs}-CT_{,S7rp/RpL32})$

2.5. Determining the developmental stages of expression of the microRNAs

RNA was extracted from different developmental stages of *A. aegypti* (larvae 1st instar, 4th instar, pupae, adult male and adult female) and *D. melanogaster* (larvae 1st instar, 3th instar, pupae, adult male, and adult female), using a Qiagen RNeasy RNA extraction kit and cDNA was produced as described in section 2.4.

The cDNA was then used to assess relative expression levels of (miR-34 and miR-124) in *A. aegypti* and *D. melanogaster,* using qRT-PCR. The reaction was performed in triplicate on a BioRad iQ5 Real-Time PCR Detection System using 96-well plates with 20 µl as described in section 2.4. For this experiment, only miR-34 primers and miR-124 primers (see Table 2.1) were used, along with ribosomal *S7* primers and ribosomal *RpL32* primers see section 2.4, which were used as an internal reference to compare relative levels of gene expression. Analysis of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) as described in section 2.4.

2.6. Preparation of Plasmids for microRNA Functional Analysis

2.6.1. Isolation of microRNAs and predicted miRNA target gene fragments (3'UTRs)

Genomic DNA was isolated from approximately 10 flies of *A. aegypti* and *D. melanogaster* using the ethanol precipitation method (Shokralla *et al* 2010). Primers were designed to isolate the 3`UTRs of *aae-porin*, *dme-porin*, *aae/014067*, and *dme/CG8292*. To isolate the gene encoding miR-34 in the both insects, extended primers were designed to amplify the precursor sequence of the miR-34 plus 100 bp of flanking sequence on both ends of the stem loop. Primer sequences are listed in Table (2.2). PCR products were resolved by electrophoresis on a 2% agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a QIAquick Gel Extraction Kit and cloned into the pSTBlue™ (Novagene) cloning vector according to the manufacturer's instructions.

Subcloning Efficiency™DH5α Chemically Competent *E. coli* cells (Invitrogen) were transformed with ligated plasmids using a heat-shock method as described by the manufacturer. The cells were then plated on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) plates with ampicillin (50 mg /ml). Transformed bacterial colonies were checked for the presence of PCR fragment inserts using a PCR colony screening method where bacterial colonies are picked and dotted into PCR tubes, then microwaved for 2 min to lyse cells. The lysed cells are then used as template in a PCR reaction similar to that used above, but scaled down to 15µl. For these reactions, T7 (5' TAATACGACGACTCACTAGGG) and Sp6 (5'

GATTTAGGTGACACTATAG) primers were used to determine if plasmids within the bacteria contained the desired insert, and an annealing temperature 42Cº was used during the PCR cycles. PCR reactions were resolved on 1% agarose gel to identify any colonies containing plasmids with appropriate-sized inserts. The bacteria colonies were then grown in 5 ml of LB broth with ampicillin (50ng/ml) overnight at 37ºC with shaking at 250 rpm to give adequate aeration to the cells. The plasmid DNA was purified from bacteria using a QIASpin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

Table 2.2. Primers used for isolation of miRNAs and predicted miRNA target gene fragments (3'UTRs) from genomic DNA. (F = forward primer, R = reverse primer).

miRNA/3`UTR	Sequence(5`-3`)	Product Size
aae-miR-34	F: TCGAGGATCCCTGCCCGAAGAACGACACAAG R: TCGAGCTAGCGAACCCGATGAACCTGGCACC	258bp
dme-miR-34	F: TCGAGGATCCCAAGATGGTGAAGCTAAAGCG R: TCGAGCTAGCCTGCCATAACCATCTGATACA	349bp
3`UTR-aae-porin	F: TCGAGGTACCGCGAGTCAAAATCGTGTTCGT R: TCGAGGGCCCTCTAGTTTTAAGGACTGTACT	342bp
3`UTR-dme-porin	F: TCGAGGTACCGTCCGTGGTTTTCCGCTAGTC R: TCGAGGGCCCTCTCAGGTAAATGCACAACGA	641bp
3`UTR-aae/014067	F: TCGAGGTACCCTTCAACTGCCCCACAAAGT R: TCGAGGGCCCTGAACACGTTTTTCCTGCAA	231bp
3`UTR-dme/CG8292	F: TCGAGGTACCGTTAAAGCTGTGGGGAAGCA R: TCGATCTAGACTATGCGCCCTACAGCAAAT	372bp

The identity of the sequences was analyzed by the Robarts Research Institute DNA Sequencing Facility (Toronto, ON), then compared them to the genome databases available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and *Aedes aegypti* genome database at the VectorBase website (http://www.vectorbase.org/) using the basic alignment search tool (BLAST. Alignments of sequences were performed using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) online alignment program ClustalW2) (http://www.ebi.ac.uk/Tools/clustalw2/).

2.6.2 MicroRNAs and target genes cloning and ligation into expression vectors

3`UTR fragments and the miRSelect™ pMIR-GFP reporter vector were digested with *KpnI* and *Apa*I or *Xba*I restriction enzymes (Fig. 2.1). MiR-34 and the miRSelect™ pEP-miR expression vector were digested with *Bam*HI and *Nhe*I restriction enzymes (Fig. 2.1). Fragments were resolved by electrophoresis on a 1% high-resolution agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a Gene JET Gel Extraction Kit according to the manufacturer's instruction. 3`UTR and miRNA fragments were ligated into pMIR-GFP and pEP-miR vectors respectively using T4 DNA Ligase (Invitrogen). Ligated vectors were then transformed into Subcloning Efficiency™ DH5α™ (Invitrogen) chemically competent cells according to the manufacturer's instruction. Vectors were isolated using a QIAprep® Spin Miniprep Kit (QIAGEN) and sent for DNA sequencing to the Center for Applied Genomics (Toronto, ON).

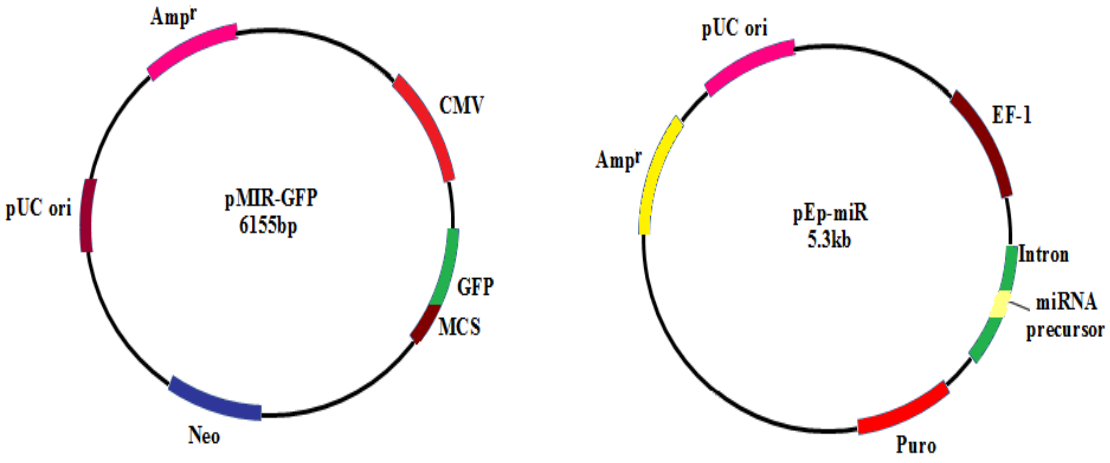


Figure 2.1. Schematic representation of pMIR-GFP reporter vector and pEP-miR cloning and expression vector.

2.6.3. MiRNA functional assays in HEK293 cells

Human Embryonic Kidney 293 cells (HEK293 cells) were maintained in DMEM 10% FBS media (+4.00mM L-Glutamine, 4500 mg/L Glucose, and 1 mM Sodium Pyruvate) at 37°C in a 5% constant flow CO_2 incubator. Cells were evenly aliquoted into wells of a 96 well plate and grown until approximately 95-99% confluent. Cells were transfected with pMIR-GFP-3'UTR+pEP-miR-34 along with negative controls; pMIR-GFP-3`UTR + pEP-miR-null and pMIR-GFP-3`UTR + pEP-has-miR-941 as well as non-transfection treated cells. Transfections were performed in triplicate with 0.2 µg vector with 0.5µl Lipofectamine™ in 100µL OptimMEM® reduced serum medium in each well. The transfection medium was changed after 4 hours. After 24 hours, transfected cells were treated simultaneously with puromycin (2µg/ml) to select for cells containing the

pEP-miR plasmid and with neomycin (G418 Sulfate) (2μg/ml) to select for cells containing the pMIR-GFP vector. GFP fluorescence expression within the cells was recorded after 24 hours of vector selection (see below).

Cells were washed with PBS and covered with 100μL PBS to facilitate measurements of GFP fluorescence. The fluorescence was read using a BioTek® microplate reader using Gen5^{TM} 1.09 software at 485nm excitation and 528nm emission, and the cell density was determined by reading the same plate at 600nm. The fluorescence of each well was normalized to blank wells containing only 100μL PBS.

2.7. Double -stranded RNA synthesis

The *aae/014067* gene fragment was excised from the pSTBlueI plasmid using two restriction enzymes, *ApaI* and *PstI,* and then ligated to the dual T7 vector pL4440 (Fig. 2.2), using T4 ligase (Invitrogen). The convergent T7 promoters within pL4440 allows for *in vitro* transcription of dsRNA. The ligated plasmid DNA was used to transform DH5α cells as described above, and putative transformed bacteria colonies were PCR screened (using pL4440 specific primers: pL4440Fwd: 5` CCACCTGGCTTATCGAA and pL4440Rev: 5` TAAAACGACGGCCAGT and the annealing temperature of 53°C in the PCR cycles) for the appropriate-sized insert. DNA sequencing was performed to confirm that the gene of interest was in the pL4440 plasmid by the Center for Applied Genomics (Toronto, ON), and sequence identity was confirmed using VectorBase's BLAST program.

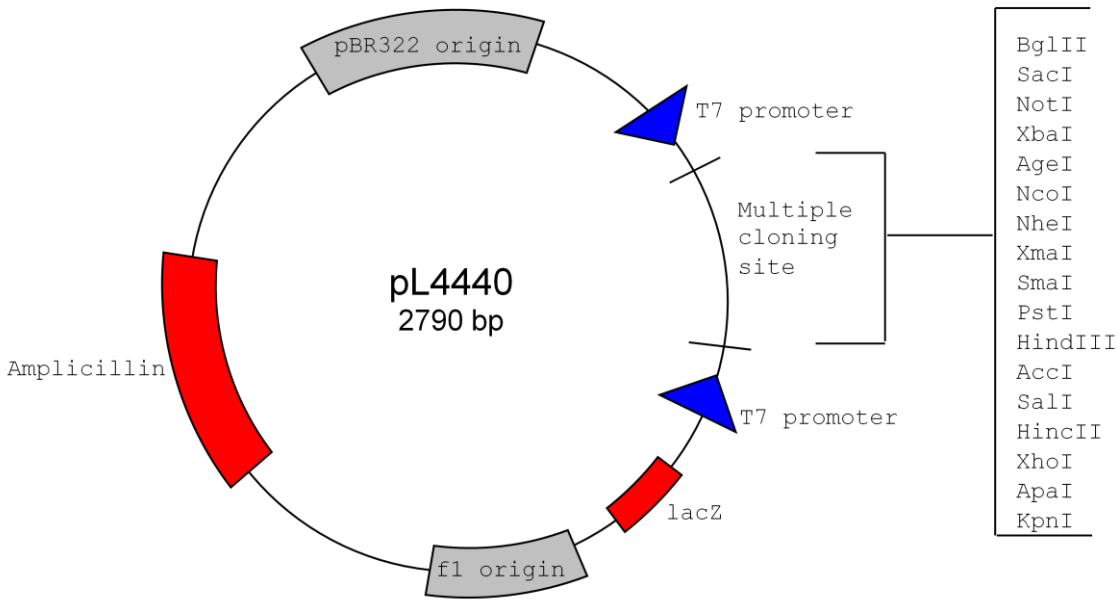


Figure 2.2. Plasmid map of the dual-T7 vector, pL4440 (Singh, 2010)

PL4440 containing a 400 bp *gus* gene fragment (as a control) was kindly provided by Cassidy Erdelyan. To obtain sufficient DNA template for *in vitro* transcription from each of the pL4440 plasmids, two standard 50 µl PCR reactions were prepared for each gene (using the following program: 94°C for 3 min, followed by 35 cycles of a 94°C for 30 sec. 52°C for 30 sec, 72°C for 2 min, followed by a final extension of 72°C for 10 min). Specially designed pL4440 primers (see section 2.9.1) were used to amplify *aae/014067* and *gus*. To check for proper PCR amplification, 10 µl of each PCR reaction was resolved on a 1% agarose gel. The remaining 90µl of the PCR reactions for each gene were then pooled together and purified using Qiagen's QIAquick PCR Purification kit to remove unused PCR reagents such as excess primers and dNTPs. The purified PCR template (1µg) of *aae/014067* and *gus* was used in 20 µl *in vitro* transcription

reactions with the MEGAscript® RNAi kit (Ambion) according to the manufacturer's specifications to produce concentrated dsRNA complementary to each gene of interest. Briefly, this kit synthesizes complementary single-stranded RNA (ssRNA) by incubating the template, along with NTPs, buffer, water and T7 RNA polymerase at 37°C for 2-4 hr, depending on the size of the gene of interest. Four hour incubations were used for *aae/014067* and *gus* dsRNA synthesis.

The complementary strands were then annealed by first denaturing the strands at 75°C for 5 min, then allowing the reactions to gradually cool to room temperature. The dsRNA reactions were then treated with DNAse to remove the template, and RNAse to remove any ssRNA, and then finally purified using a filter column. A 10-fold diluted sample of purified dsRNA was resolved on a 1% agarose gel to check for size and purity of the sample. Concentrations of dsRNA were measured using a NanoVue spectrophotometer (GE Healthcare).

2.8. Antisense oligonucleotide design

The mature sequence of *A. aegypti* miR-34 was taken from miRBase and uploaded into Gene Tools software (www.**gene-tools**.com/) to design an antisense morpholino with the following sequence: ACACAACCAGCTAACCACACTGCCA. An antisense morpholino was designed using Integrated DNA Technologies (IDT) (www.**idt**dna.com/) software. The IDT (*Aamir34m*) oligonucleotide is a 2′-O-Me/DNA phosphorothioate chimera oligonucleotide; mA*mC*mA*mC*A*A*C*C*A*G*C*T*A*A*C*C*A*C*A*C*T*mG*mC*mC*mA*/3Phos/, where "mX" refers to O-methyl ribonucleotides and "*X" refers to

phosphorothioate deoxribonucleotides. Binding of this chimeric oligonucleotide to its target will induce RNase H-mediated cleavage of the target miRNA (Grünweller *et al*., 2003).

2.9. Mosquito injections and mating bioassay

aae/014067 dsRNA was diluted to 350 ng/µl in water before being injected into mosquitoes, as this concentration has been previously observed to induce RNAi in mosquitoes (Erdelyan *et al*, 2010), while the *Aamir34m* antisense oligonucleotide was diluted to various concentrations (200 µM, 40 µM, 10 µM, and 1µM) prior to injection. Injections were performed using borosilicate glass needles that were prepared with a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments).

Approximately 75-100 nl of *aae/014067* dsRNA, *gus* dsRNA, and *Aamir34m* were injected into the thorax of male mosquito pupae and adults. After the injections, mosquito adults were placed in plastic vials, while pupae were placed in plastic vials containing dechlorinated tap water plus ampicillin (final volume, approx. 20 µg/ml). The insects were then observed for survival, and survivors were provided virgin females to assess their ability to reproduce. Three days after mating, the females were provided blood and then allowed to lay their eggs. The number of eggs and the percentage hatch were recorded.

Subsets of injected adults were collected 2 days after injection to testing for RNAi efficiency. Insects were pooled and placed in -80ºC until RNA extraction and cDNA synthesis. Knockdown of the *aae/014067* and aae-miR-34 was determined using qRT-PCR as described above. *S7rp* primers were again used to amplify the reference gene, and

a new set of *aae/014067* primers were designed as follow: aae/014067F: 5`

GGACGAAATTGGAGTCGGAATA and aae/014067R: 5`

GCAGTTGAAGATCCAGGAAGTA, amplifying a 98 bp product.

3. Results

3.1. Identification and characterization of candidate microRNAs

Subsets of miRNAs have been identified in mice and humans that are expressed either exclusively or preferentially in the testis. Putative homologues of these miRNAs were found in the genome databases of *A. aegypti* and *D. melanogaster* (Table.3.1). The miRNAs selected for this study were those with the highest sequence identities to the human or mouse miRNAs. Using these criteria, five miRNAs with percentage identities of 95% or higher to the mammalian miRNAs were found in both the mosquito and vinegar fly genomes. Four of the five mature miRNA sequences were identical in the two insect species, while the fifth miRNA (miR-100) was 95% identical in the two insects, which suggests that these miRNAs may have similar functions in the two insect species, and possibly shared functions to those found in the mammalian species.

Table.3.1. Candidate testis miRNAs in *A. aegypti* and *D. melanogaster*. The percent identities between mature (i.e. fully processed) miRNAs sequences in *A. aegypti* and *D. melanogaster* are indicated.

miRNA name	***Aedes aegypti* Sequence (5′–3′)**	**Mammalian orthologue***	**% Identity between *Aedes* (aae) and *Drosophila* (dme)**
miR-9	UCUUUGGUUAUCUAGCUGUAUGA	mmu-miR-9	100%
miR-34	UGGCAGUGUGGUUAGCUGGUUG	hsa-miR-34	100%
miR-100	AACCCGUAGAUCCGAACUUGUG	mmu-miR-100	95%
miR-124	UAAGGCACGCGGUGAAUGC	hsa-miR-124	100%
miR-219	UGAUUGUCCAAACGCAAUUCUUG	mmu-miR-219	100%

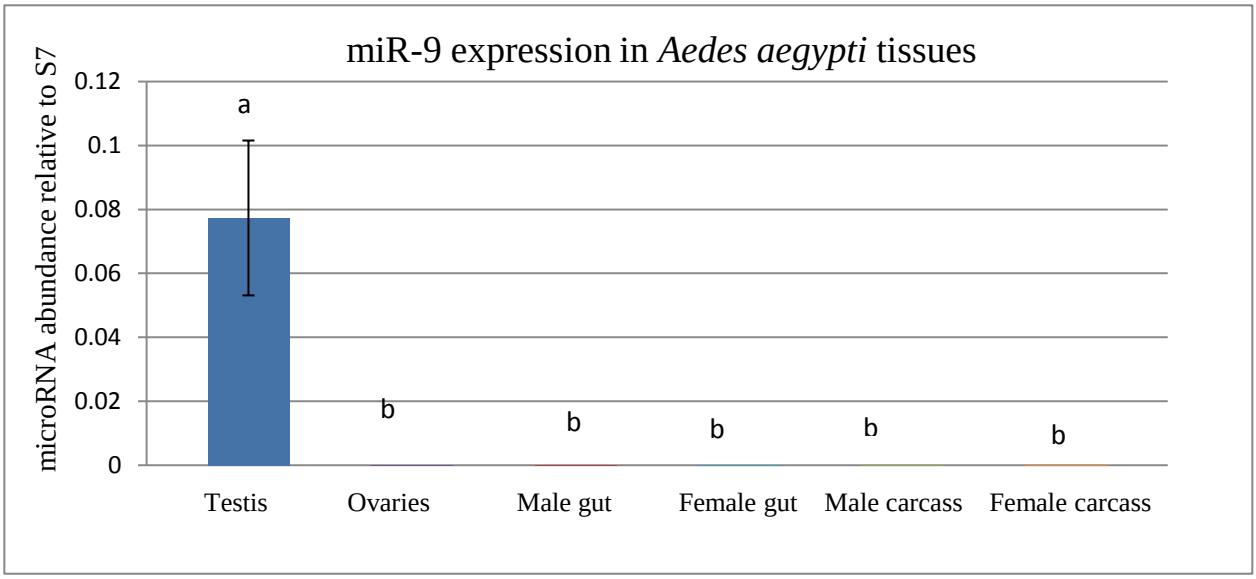
* mmu – mouse miRNA; hsa – human miRNA

3.2. Tissue-specific and sex-specific expression profiles of microRNAs

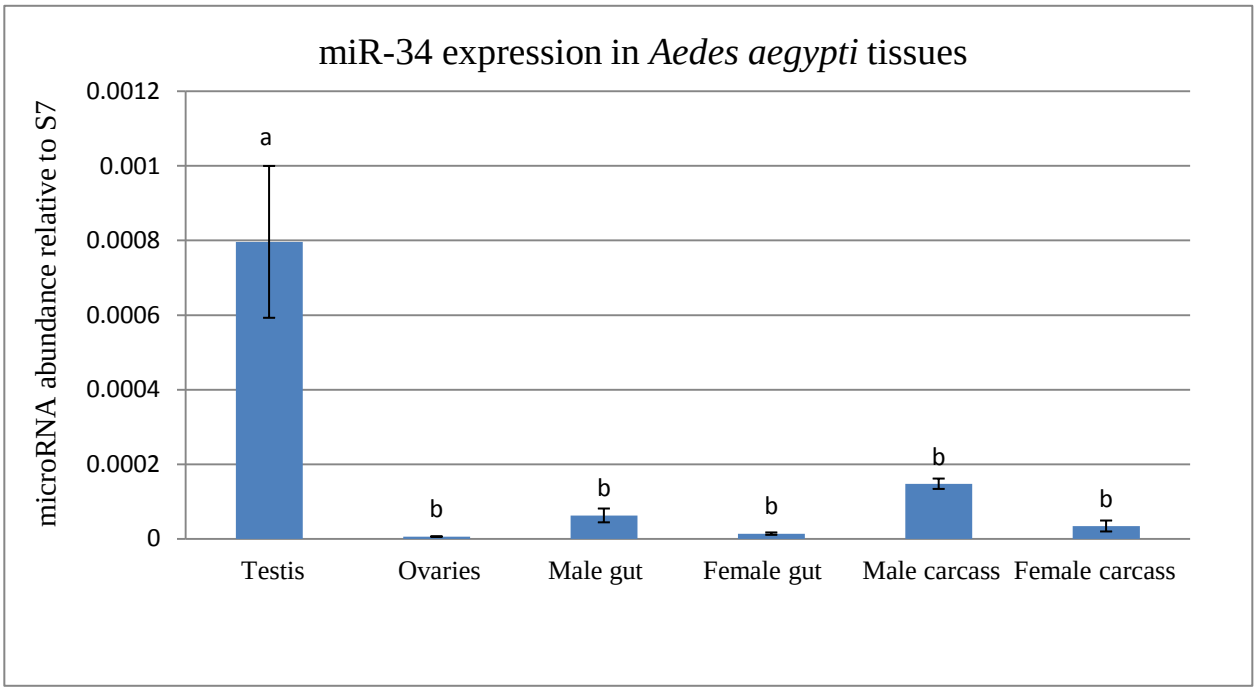
The expression of the selected miRNAs in different tissues and the two sexes was examined using qRT-PCR with cDNAs derived from various tissues in both male and female *A. aegypti* and *D. melanogaster*. In the mosquitoes, only one of the five miRNAs examined, miR-9, was exclusively expressed in the testis (Fig. 3.1A). MiR-34 transcripts were approximately 4 times more abundant in the testis than any other male tissues, and were more than 20 times more abundant in testis than ovaries (Fig. 3.31B). The other three miRNAs, (miR-100, miR-124, and miR-219) all showed male-biased expressions in some, but not all tissues, but they were not testis-specific (Fig.3.1C, 3.1D and 3.1E respectively).

Figure 3.1

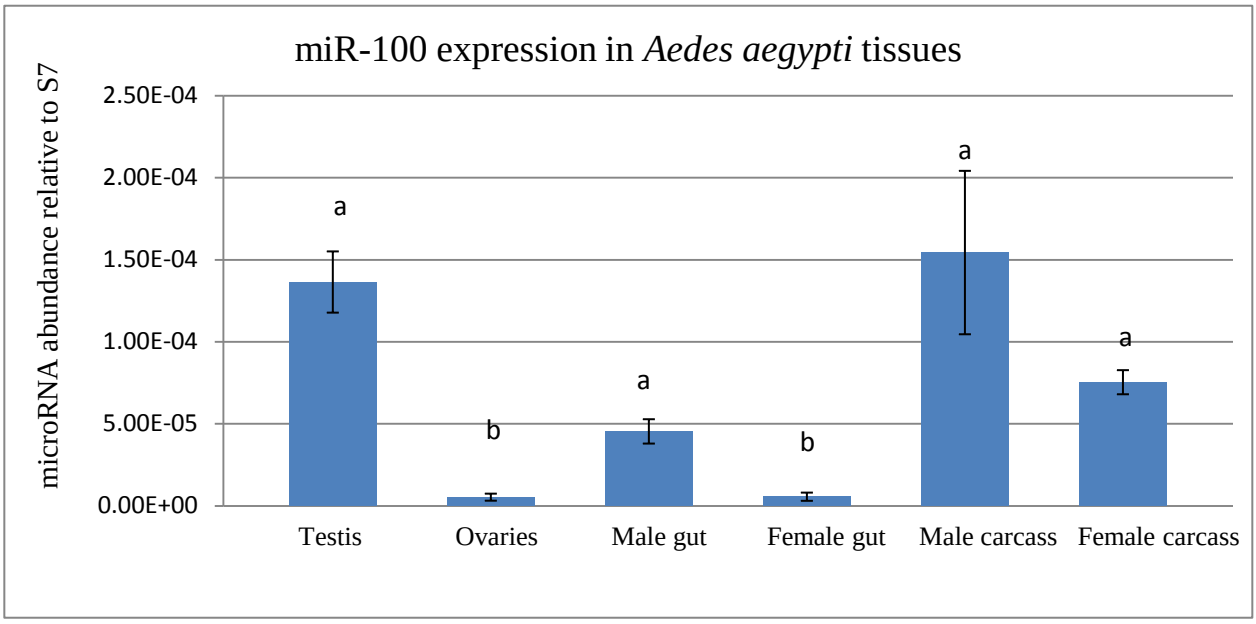
A



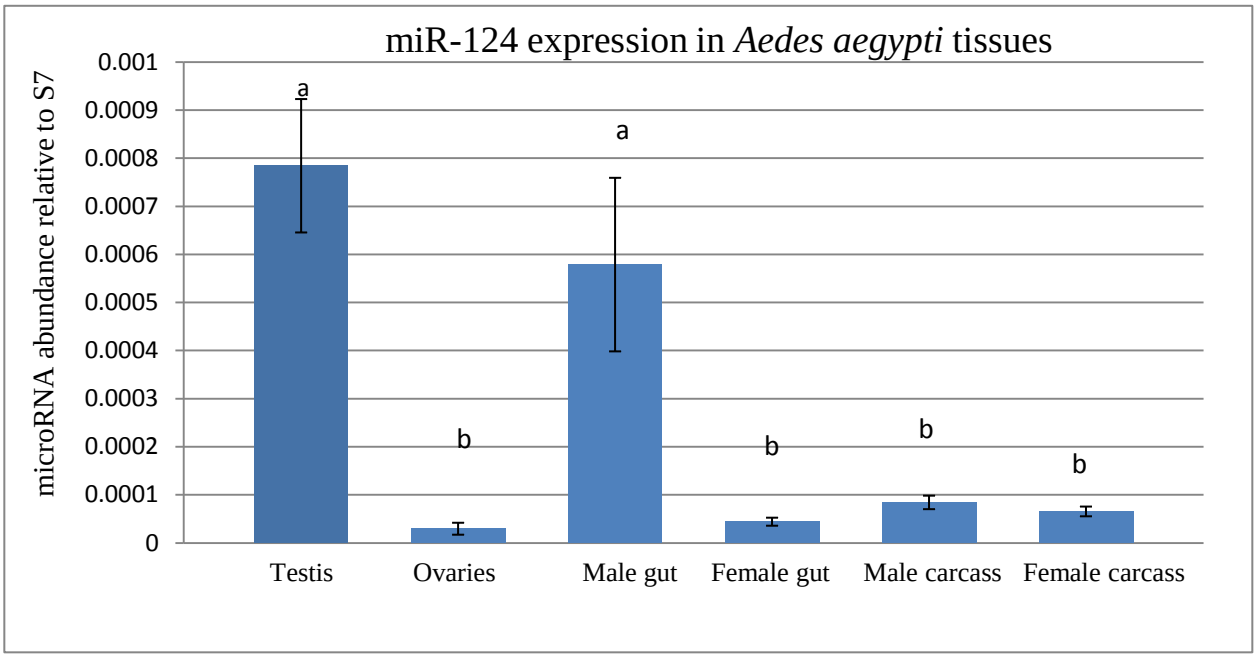
B


miR-34 expression in *Aedes aegypti* tissues
microRNA abundance relative to S7
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
a
b
b
b
b
b
Testis
Ovaries
Male gut
Female gut
Male carcass
Female carcass

C


miR-100 expression in *Aedes aegypti* tissues
microRNA abundance relative to S7
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
a
b
a
b
a
a
Testis
Ovaries
Male gut
Female gut
Male carcass
Female carcass

D



E

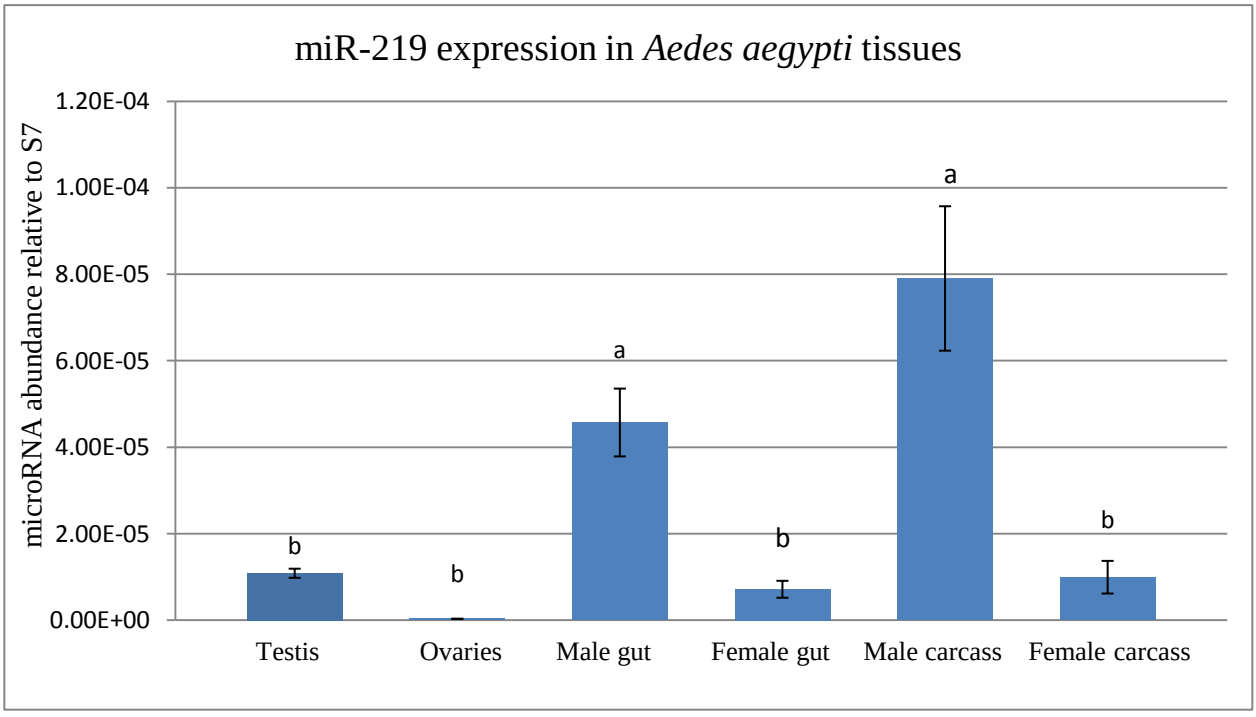
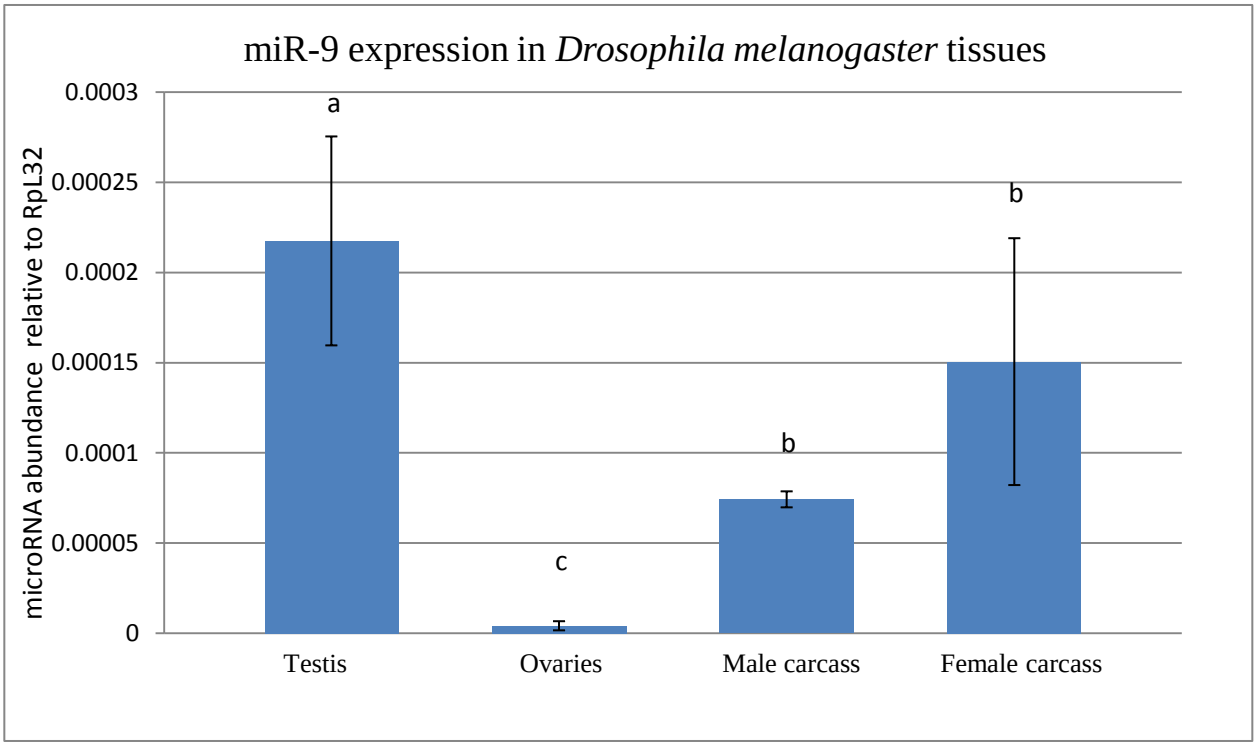


Figure 3.1. Expression levels of miR-9 (A), miR-34 (B), miR-100 (C), miR-124 (D) and miR-219 (E) in *A. aegypti* tissues relative to ribosomal protein gene *S7*. Values represent the mean and standard error of mean of three biological replicates. Different letters above the bars reflect significantly different expression levels (ANOVA, $P<0.05$).

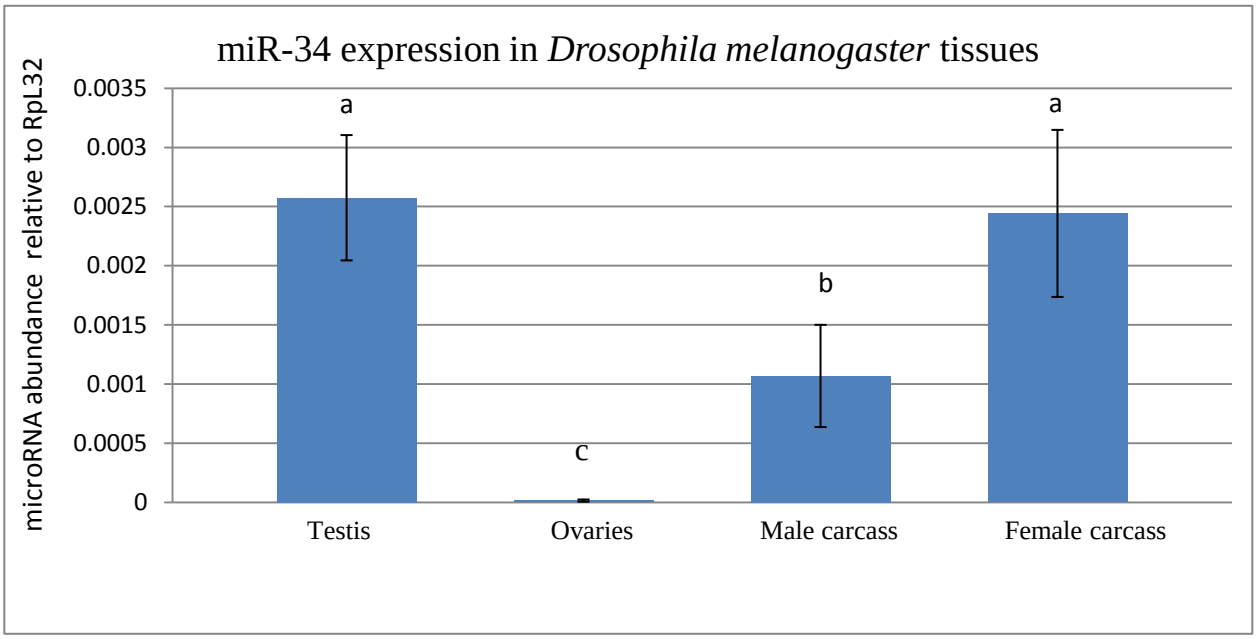
In *Drosophila*, the tissue-specificity of the miRNAs showed both some similarities and differences from the tissue-specificity observed in the mosquito. Like the expression in *A. aegypti*, miR-9 and miR-34 in *D. melanogaster* showed higher expression in the testis relative to the rest of the body (Fig 3.2A and 3.2B), but while miR-9 was exclusively found only in males in mosquitoes, in *D. melanogaster* it was also expressed in female ovaries and the carcass. MiR-100 showed similar patterns of expression in both insects, with slightly more expression in the testis, but also expressed in the male and female carcass (Fig 3.2C). MiR-124 was apparently male-specific in the mosquito, but in *D. melanogaster*, it was also detectable in the female carcass (Fig 3.2D). In contrast to the non-testis-specific expression of miR-219 in *Aedes*, this miRNA in *Drosophila* appears to be exclusively expressed in the testis (Fig. 3.2E).

Figure 3.2

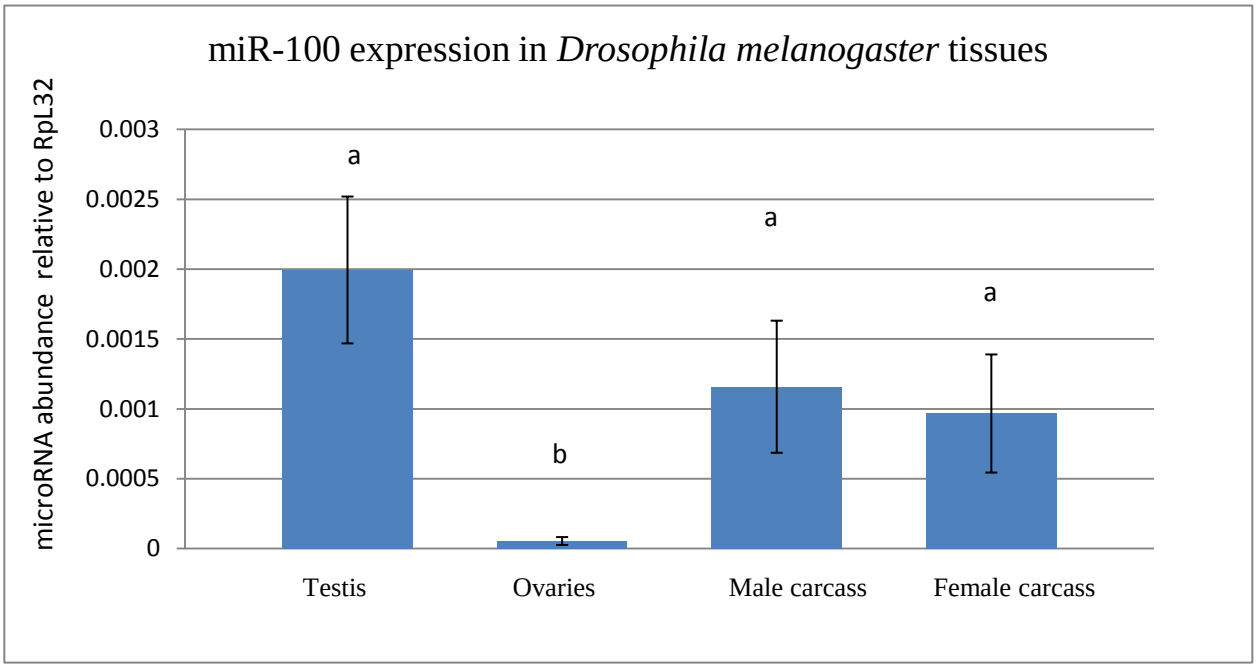
A



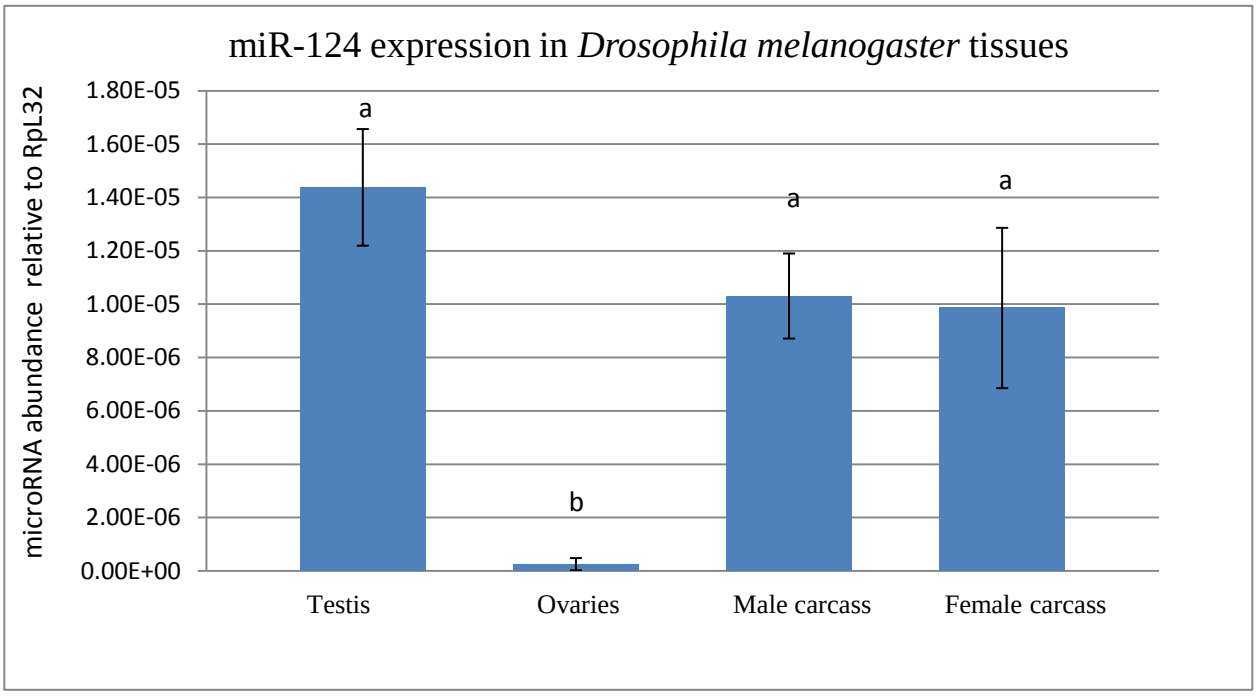
B


miR-34 expression in *Drosophila melanogaster* tissues
microRNA abundance relative to RpL32
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
a
c
b
a
Testis
Ovaries
Male carcass
Female carcass

C


miR-100 expression in *Drosophila melanogaster* tissues
microRNA abundance relative to RpL32
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
a
b
a
a
Testis
Ovaries
Male carcass
Female carcass

D



E

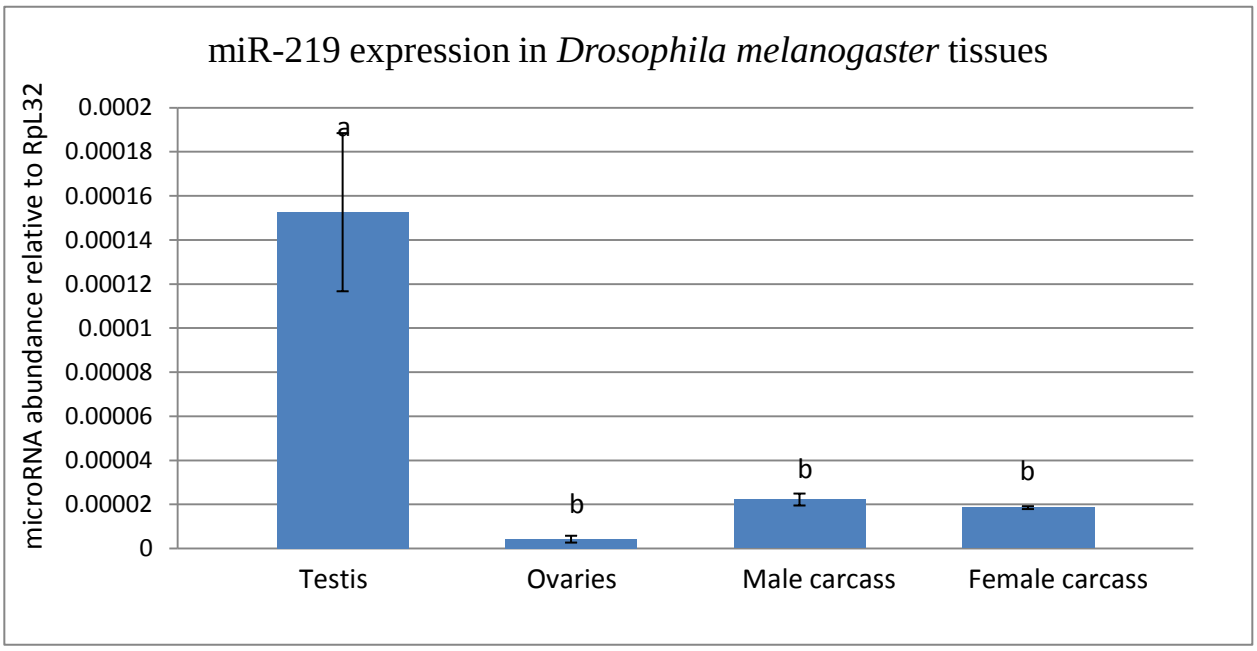


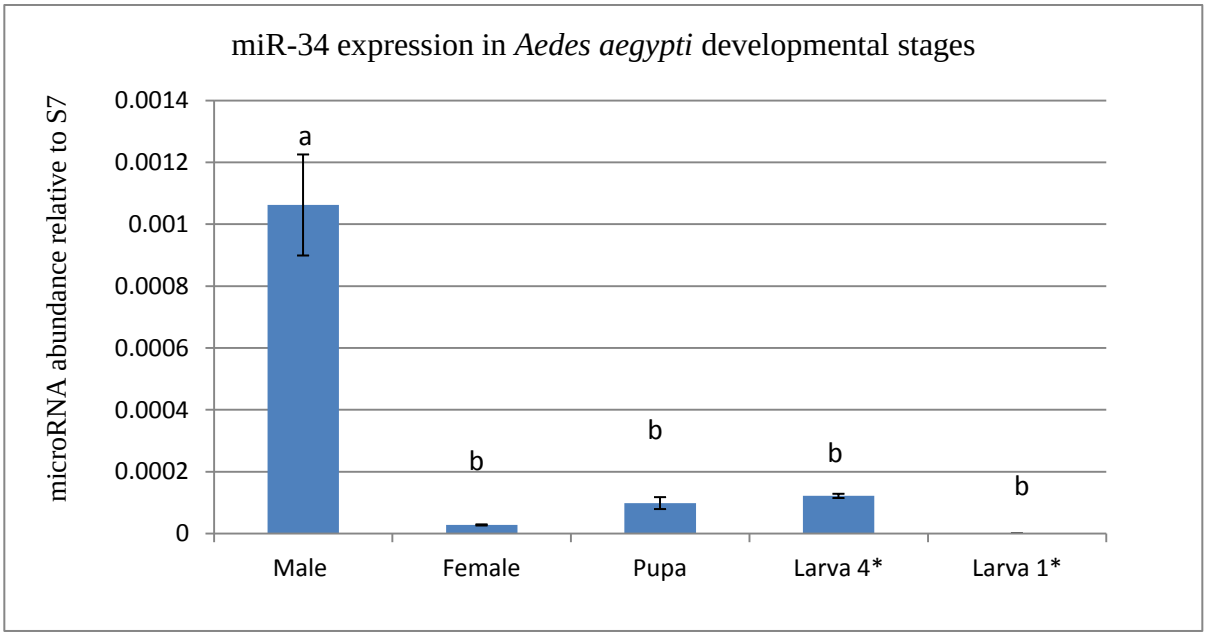
Figure 3.2. Expression levels of miR-9 (A), miR-34 (B), miR-100 (C), miR-124 (D) and miR-219 (E) in *D. melanogaster* tissues relative to ribosomal protein gene *RpL32*. Values represent the means and standard errors of the mean of three biological replicates. Different letters above the bars reflect significantly different expression levels (ANOVA, $P<0.05$).

3.3. Developmental expression profile of microRNAs

The developmental expression profile of miRNAs was examined using qRT-PCR with cDNAs derived from various life stages of *A. aegypti* and *D. melanogaster*. Two miRNAs, miR-34 and miR-124, were examined for this objective. MiR-34 was predominantly expressed in adult males, relative to any other developmental stage in both *Aedes* and *Drosophila* (Fig. 3.3A and 3.3B respectively). In the mosquito, this miRNA was expressed at least 10 times more abundantly in the adult males relative to any other developmental stage, whereas in *D. melanogaster*, it was expressed about two times greater in adult males than females. While male and female *D. melanogaster* showed similar levels of expression of miR-34 in the carcass (see Figures 3.1B and 3.2B), the testis can account for a significant mass of the sexually mature insect (White-Cooper, 2010), and hence, this tissue-biased expression likely accounts for the greater overall expression of miR-34 in male flies, relative to females. Overall, the high expression of miR-34 in testis and adult males in both *A. aegypti* and *D. melanogaster*, along with the fact that this miRNA has documented roles in mammalian germ cell and gonadal somatic cells (Bohallier *et al*., 2010; *Bao et al*., 2012; Hossain *et al*., 2012), make it interesting candidate miRNA worthy of further examination. MiR-124 was expressed primarily in pupae and adults of the mosquito, especially males, which may also indicate a testis function for this miRNA, although it likely has functions beyond this tissue in *Aedes* (Fig. 3.4A). In *Drosophila*, this miRNA was primarily expressed in pupae, which corresponds to a period of enhanced testis development and commencement of spermatogenesis (Fig. 3.4B) (Casper and Van Doren, 2006).

Figure 3.3

A



B

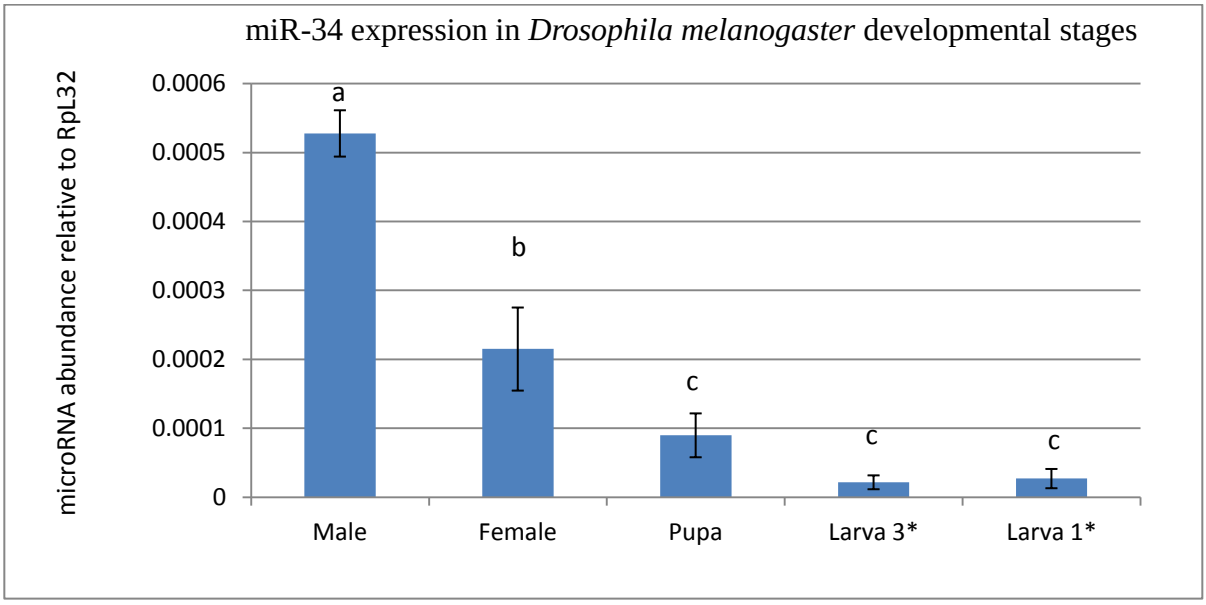
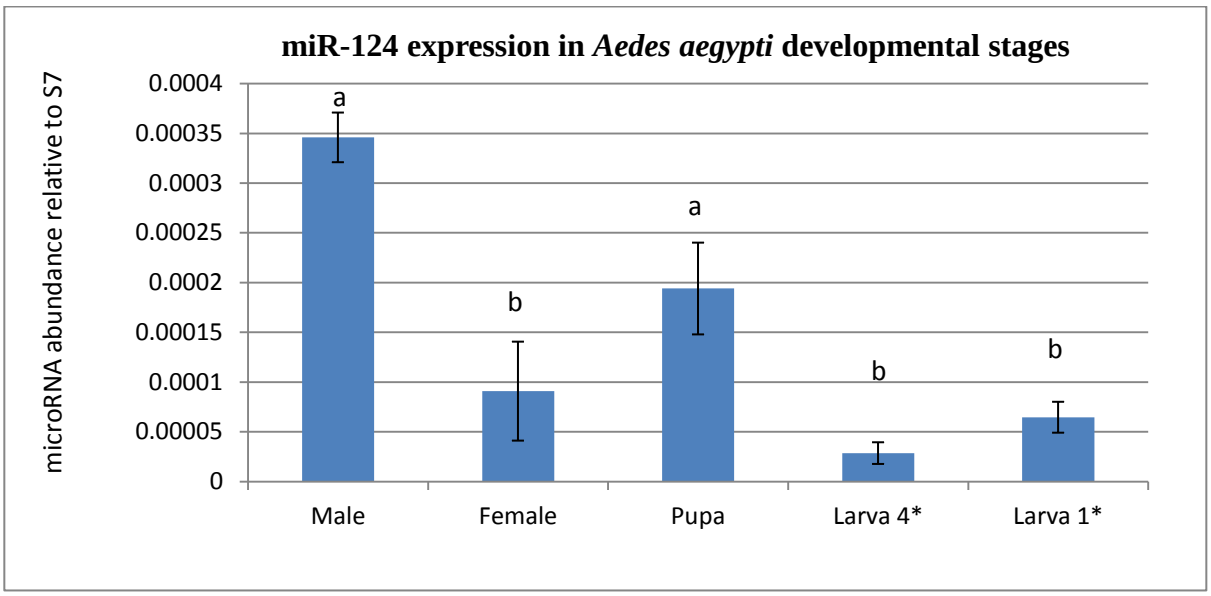


Figure 3.3. Expression levels of miR-34 in *A. aegypti* (A) and *D. melanogaster* (B) developmental stages relative to ribosomal protein gene *S7* and *RpL32* respectively. Values represent the means and standard errors of the mean of three biological replicates. Different letters above the bars reflect significantly different expression levels (ANOVA, $P<0.05$).

Figure 3.4

A



B

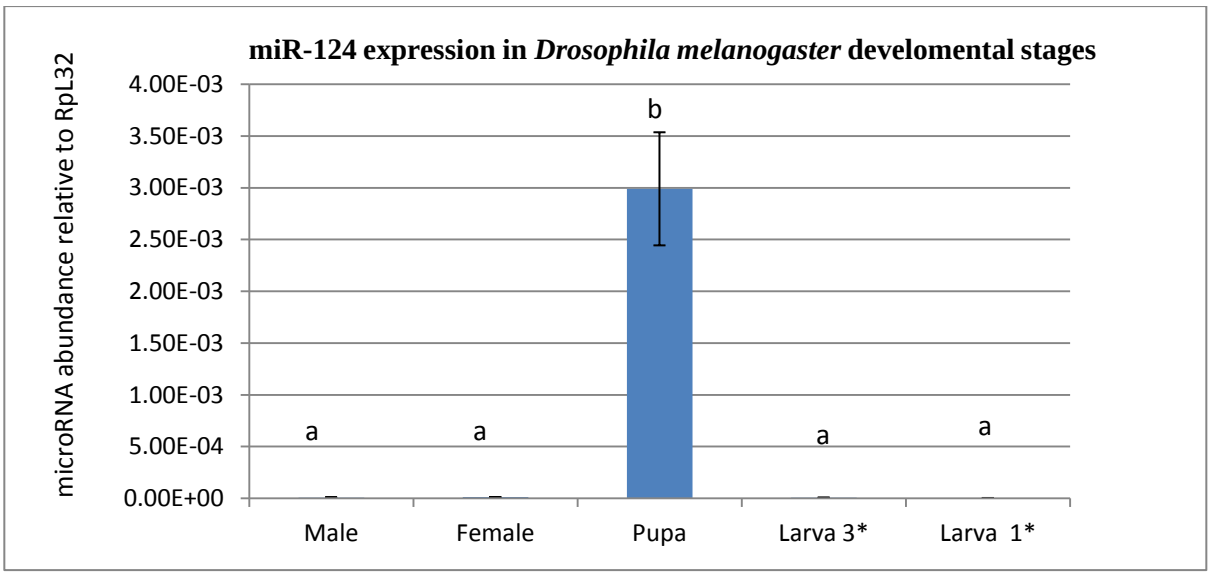


Figure 3.4. Expression levels of miR-124 in *A. aegypti* (A) and *D. melanogaster* (B) developmental stages relative to ribosomal protein gene *S7* and *RpL32* respectively. Values represent the means and standard errors of the mean of three biological replicates. Different letters above the bars reflect significantly different expression levels (ANOVA, $P<0.05$).

3.4. Identification and characterization of potential microRNA gene targets

Putative target genes for miR-34 in *A. aegypti* and *D. melanogaster* were examined and analyzed based on two criteria: genes with known function that are involved in testis development or spermatogenesis; or genes of unknown function that, based on gene expression data available at FlyBase (http://flybase.org/) or VectorBase (https://www.vectorbase.org/), have been experimentally determined to be expressed exclusively in testis tissues. Based on these criteria, four predicted miR-34 targets were identified from TargetScanFly and MicroInspector. The four predicted target genes are listed in Table 3.2.

Table 3.2. Candidate target genes of miR-34 in *A. aegypti* and D. *melanogaster*. Asterisks indicate that no known orthologues for these genes in other species have been identified

Gene Name	Gene Symbol	Function
dme-porin	CG6647	Sperm individualization
aae-porin	AAEL001872	Sperm individualization
dme/CG8292*	CG8292	Unknown
aae/014067*	AAEL014067	Unknown

In *Drosophila*, porin is involved in sperm individualization and sperm mitochondrial organization (Graham *et al*., 2010). Using BLAST, a putative homologue of porin was easily identified in the genome of *A. aegypti*, with 67.44% identity to the *D. melanogaster* gene. The putative 3`UTRs of the porin genes in each insect, comprising 500 bp of sequence downstream of the last exon, were examined for miR-34 binding site

using TargetScan and MicroInspector. The computational algorithm predicted that miR-34 binds at nucleotide position +282 bp within the 3`UTR of *dme-porin* (Fig.3.5A) (where position +1 denotes the first nucleotide following the stop codon), while the binding site in 3`UTR of *aae-porin* was at position +230 bp (Fig.3.5B).

The *dme/CG8292* gene from *D. melanogaster* and the *aae/014067* gene from *A. aegypti* show no similarity to each other, but both were previously found to be expressed exclusively in the testis of each species (Graveley *et al*., 2011; Dissanayake *et al*., 2010), but the function of these genes are unknown. No known orthologues for these two genes have been identified in any other species, but the fact that they were described as testis-specific and they were predicted to bind to miR-34 makes them interesting candidate genes. Examination of the 3`UTRs of these two testis-specific genes using MicroInspector predicted that miR-34 binds at +280 bp within the 3`UTR of *dme/CG8292* (Fig.3.5C) and at +180 bp within the 3`UTR of *aae/014067* (Fig.3.5D).

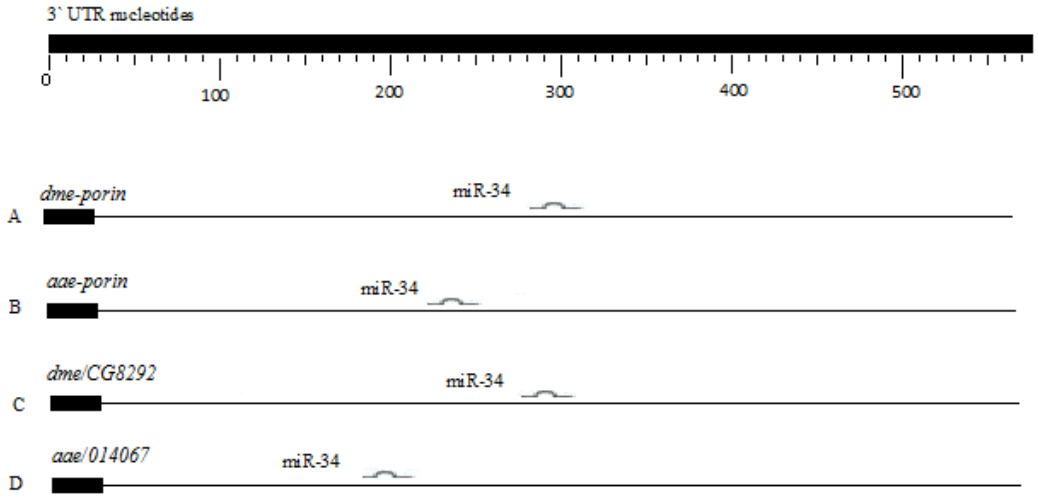
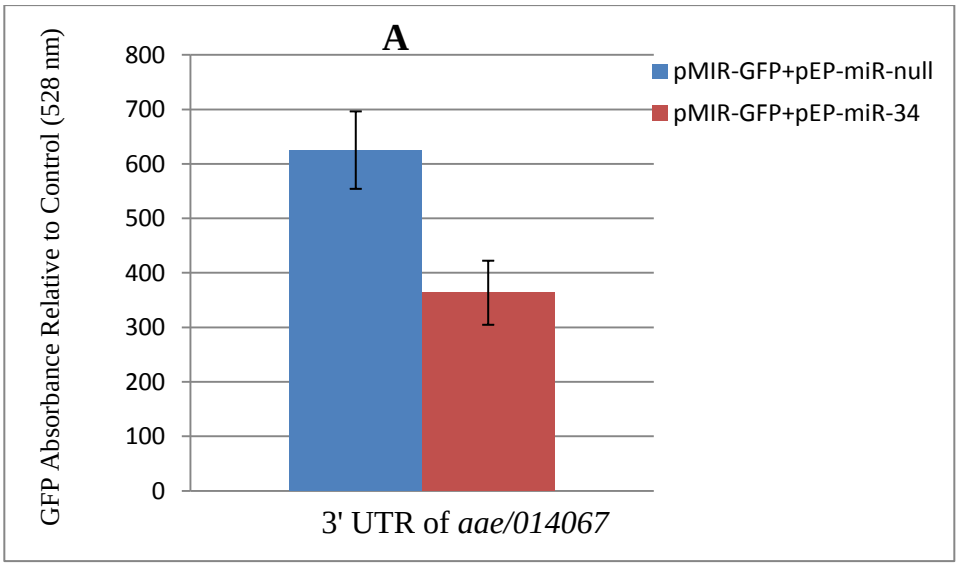


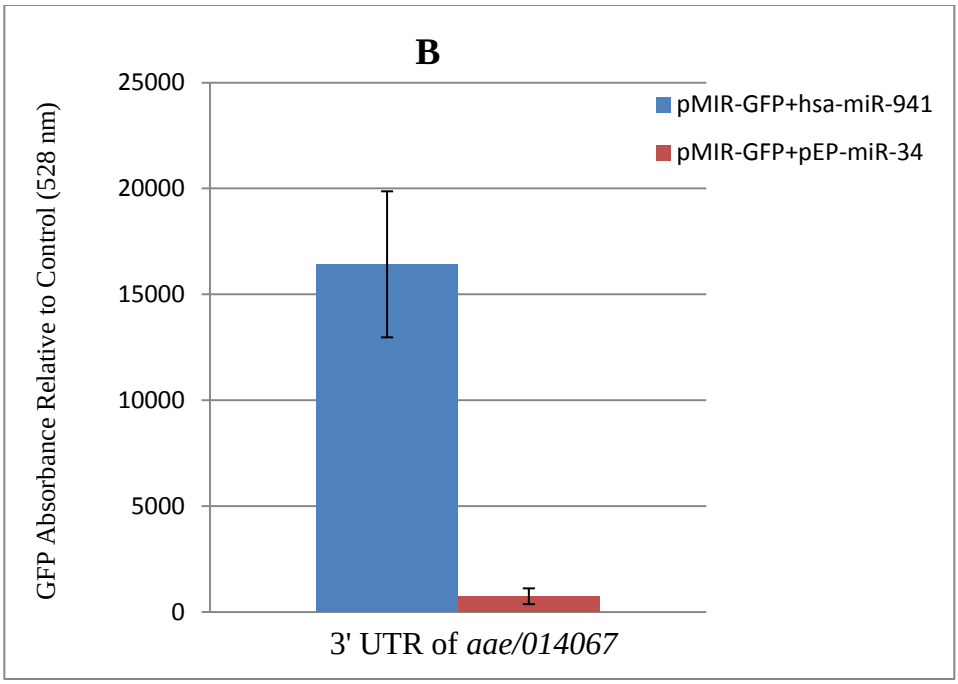
Figure. 3.5. MicroRNA- 34 target sites in the 3`UTRs of *dme-porin* (A), *aae-porin* (B), *dme/CG8292* (C), and *aae/014067* (D).

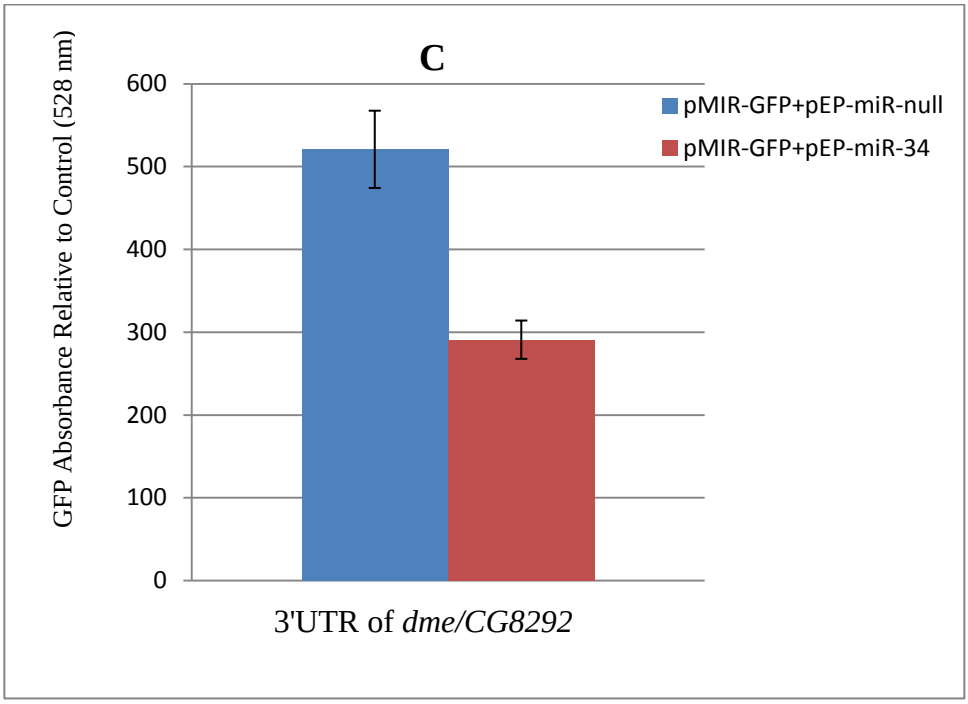
3.5. microRNAs and target genes interaction

A cell-based assay was used to assess whether miR-34 could bind to its predicted target genes (see Table 3.2). Equal dosages of the pMIR-GFP-3`UTR reporter and the pEP-miR-34 expression vectors along with two negative controls (pEP-miR-null or pEP-has-miR-941), were then co-transfected into HEK293 cells. After three days, the extent of GFP fluorescence was measured to determine whether the miR-34 could down-regulate expression of GFP derived from the pMIR-GFP-3`UTR reporter gene plasmid. In these assays, miR-34 reduced the expression the GFP reporter linked to the UTRs of *aae/014067* and the *dme/CG8292* (Fig.3.6) as well as the UTRs of the porin genes in both the mosquito and vinegar fly (Fig. 3.7). This latter result suggests that miR-34 has a conserved function in regulating porin's expression in both species. It is worth noting however, that despite this possible common function in testis gene expression, this miRNA may have different functions in other tissues in the two insects, given the differences in tissue- and stage-specificity noted above.

Figure 3.6




B
GFP Absorbance Relative to Control (528 nm)
25000
20000
15000
10000
5000
0
pMIR-GFP+hsa-miR-941
pMIR-GFP+pEP-miR-34
3' UTR of aae/014067


C
GFP Absorbance Relative to Control (528 nm)
600
500
400
300
200
100
0
pMIR-GFP+pEP-miR-null
pMIR-GFP+pEP-miR-34
3'UTR of dme/CG8292

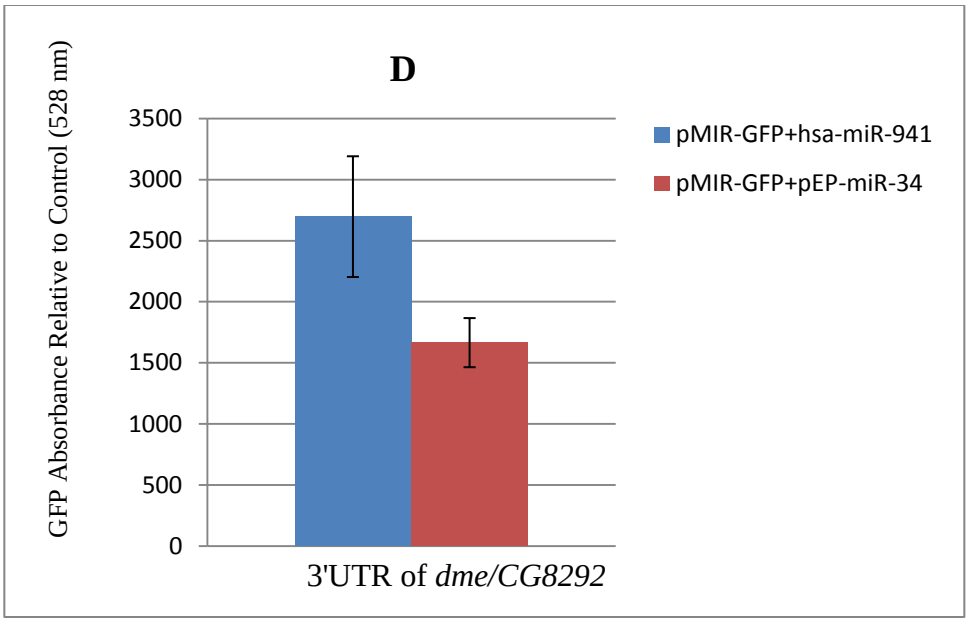
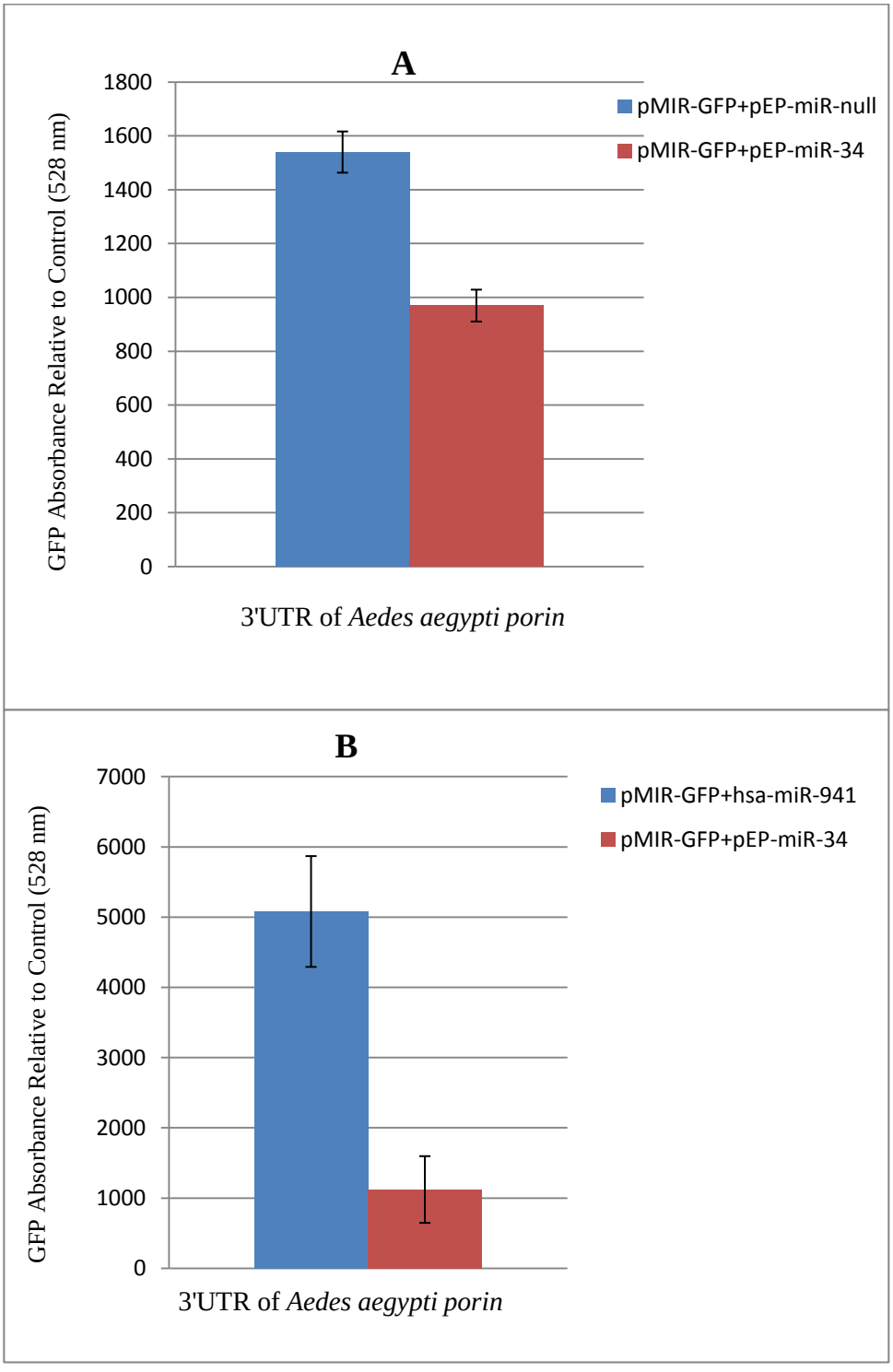


Figure 3.6. Cell based assays to assess whether the *miR-34* can bind to the *aae/014067*and the *dme/CG8292* 3`UTRs. The fluorescence levels derived from the cells transfected with pMIR-GFP- *aae/014067*-3`UTR+ pEP-miR-34 plasmids were normalized to fluorescence in cells transfected with pMIR-GFP- *aae/014067*-3`UTR+Pep-miR-null (a plasmid that expresses no miRNA) (A) and to pMIR-GFP-*aae/*014067-3`UTR+Pep-has-miR-941 (a plasmid that expresses a human-specific miRNA with no predicted binding affinity to the target UTR) (B). The pMIR-GFP-*dme/CG829*- 3`UTR+ pEP-miR-34 plasmids were normalized to pMIR-GFP-*dme/CG8292*-3`UTR+Pep-miR-null (C) and to pMIR-GFP- *dme/CG8292*-3`UTR+Pep-has-miR-941 (D). The values represent the means and standard errors for three replicate experiments. All values were significantly different from one another (student t-test; $p<0.05$).

Figure 3.7


A
1800
1600
1400
1200
1000
800
600
400
200
0
GFP Absorbance Relative to Control (528 nm)
pMIR-GFP+pEP-miR-null
pMIR-GFP+pEP-miR-34
3'UTR of Aedes aegypti porin
B
7000
6000
5000
4000
3000
2000
1000
0
GFP Absorbance Relative to Control (528 nm)
pMIR-GFP+hsa-miR-941
pMIR-GFP+pEP-miR-34
3'UTR of Aedes aegypti porin

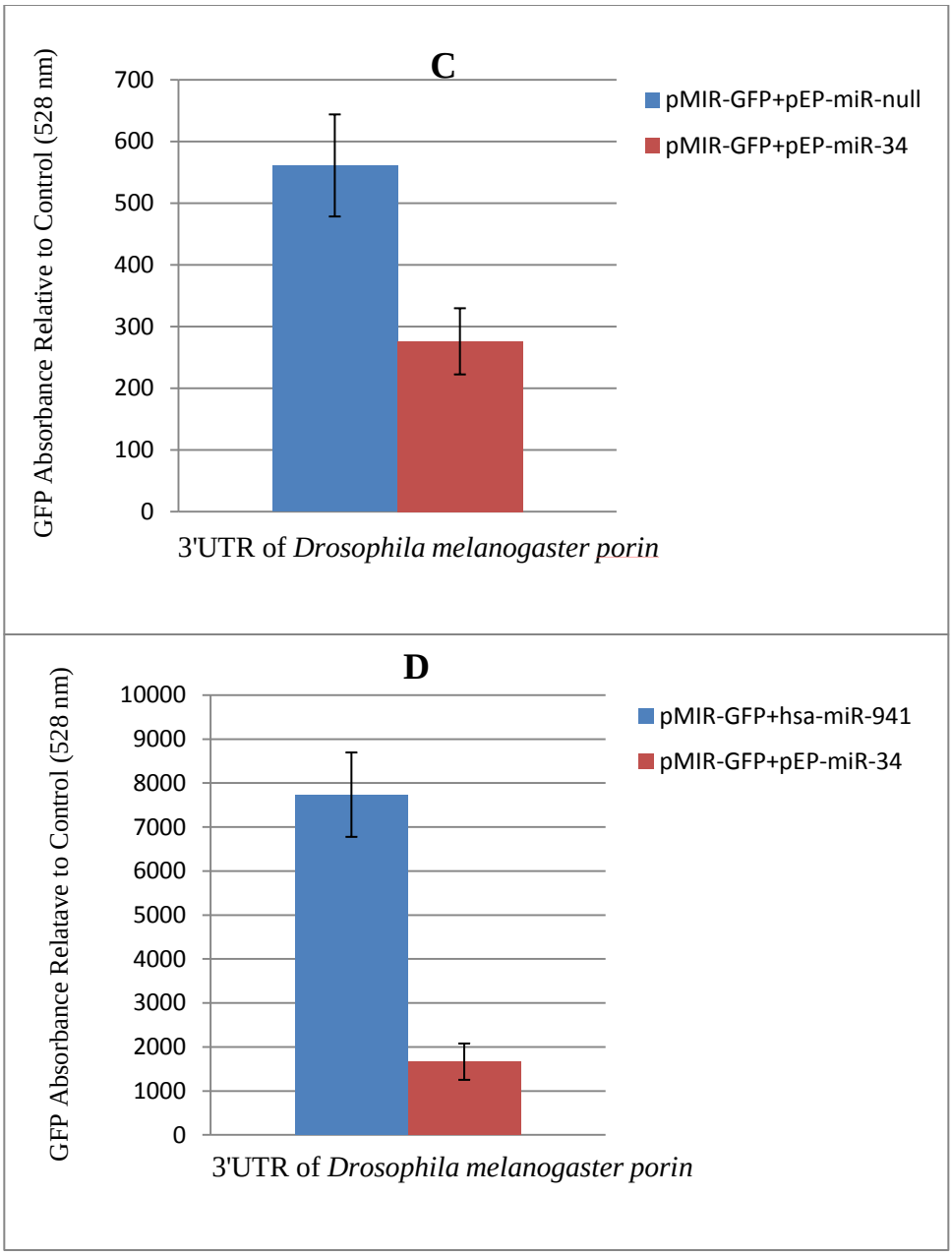


Figure 3.7. Cell based assays to assess whether the *miR-34* can bind to the *aae/porin* and the *dme/porin* 3`UTRs. The fluorescence detected in cells transfected with pMIR-GFP-*aae/porin*-3`UTR+ pEP-miR-34 plasmids were normalized to fluorescence in cells transfected with pMIR-GFP- *aae/porin*-3`UTR+Pep-miR-null (A) and to pMIR-GFP-*aae/porin*-3`UTR+Pep-has-miR-941 (B). The pMIR-GFP- *dme/porin*- 3`UTR+ pEP-miR-34 plasmids were normalized to pMIR-GFP- *dme/porin*-3`UTR+Pep-miR-null (C) and to pMIR-GFP- *dme/porin*-3`UTR+Pep-has-miR-941 (D). The values represent the means and standard errors for three replicate experiments. All values were significantly different from one another (student t-test; $p<0.05$).

3.6. Mosquito injections and mating bioassays.

To examine the roles of *aae/014067* and miR-34 in *A. aegypti* male fertility, mosquitoes were injected at both the pupal and adult stages with dsRNA targeting the *aae/014067* gene or an antisense oligonucleotide targeting miR-34. The β-glucuronidase (*gus*) dsRNA served as a negative control for the RNAi experiment, as the *gus*-gene shows no homology to *A. aegypti* genes and has been used in previous studies of RNAi in other insects (Whyard *et al*., 2009).

Injection of the *aae/014067* dsRNA caused significant mortalities in pupae, relative to the negative control injections; approximately 89% of the pupae injected with the *aae/014067* dsRNA died, while only 8% of the gus-dsRNA-injected pupae died (Table 3.3). This high mortality suggests that the *aae/014067* dsRNA was either cross-silencing other genes essential for pupal or early adult development, or that the target gene is not necessarily restricted to the testis, but is expressed in other tissues that require its proper expression to ensure a normal metamorphosis from pupa to adult. An initial search for possible cross-silencing of other genes was performed by using BLAST to search for regions of 21 nt or more matching, and none were detected. It is however, possible that the dsRNA could be targeting other genes with imperfect complementarity. To circumvent the pupal mortalities, freshly eclosed (<6 h old) adult males were then injected with the *aae/014067* dsRNA, and mortalities were reduced to levels comparable to that observed using the *gus*-dsRNA (Table 3.3). The improved survival suggests that the *aae/014067* dsRNA was not toxic to the adult mosquitoes. Interestingly, when the surviving males were mated with virgin females, their fecundity was observed to be reduced by more than 95%, relative to the negative control mosquitoes that were injected

with the *gus* dsRNA (Table 3.4). While the actual role of the gene is still not fully resolved, it appears to be associated with male fertility.

Table 3.3. Mortality and surviving success of individual male *A. aegypti* injected with dsRNA.

Life stage injected	dsRNA injected	Total injected	Surviving injectants	% mortality 1-2 day after injection
Pupae	*gus*	38	35	7.9%
	aae/014067	45	5	88.9%*
Adults	*gus*	35	33	5.7%
	aae/014067	40	36	10%

* Significantly greater mortality than the gus-dsRNA negative control (Fisher's exact test, $p<0.05$).

Table 3.4. Fecundity data obtained from mated injected males with uninjected females at the adult stage.

Treatment	# male adults injected	# blood-fed females	Total eggs hatched	# viable eggs/mated female
gus-dsRNA	33	15	63	4.2±1.7
aae/014067-dsRNA	36	13	2	0.15±0.16*

***** indicates significant difference in number of viable eggs per mated females related to negative control (Fisher's exact test, $p<0.05$).

Mosquito pupae injected with different dosages of the *Aamir34m* antisense oligonucleotide also showed high mortality, as 10 µM, 40 µM and 200 µM of antisense oligonucleotides induced between 80% and 100% mortality compared to the negative controls that were injected with injection buffer, whereas 1 µM injected pupae and 40 µM injected adults showed no significant difference in mortality related to the negative

controls. (Table 3.5). The fact that the *Aamir34m* antisense oligonucleotide affected the survival of pupae suggests that this miRNA is essential for pupae survival and development. This is not very surprising, as miR-34 is predicted to bind to many genes, many of which are genes essential for development (Table.3.6).

Table 3.5. Mortality and surviving success of individual male *A. aegypti* injected with *Aamir34m* oligonucleotide.

Life stage injected	Oligonucleotide concentration	Total injected	Surviving injectants	% mortality 1-2 day after injection
Pupae	Control	13	13	0%
	1 µM	5	4	20 %
	10 µM	5	1	80%*
	40µM	10	0	100%*
	200µM	20	0	100%*
Adults	Control	26	25	3.85%
	40µM	32	28	12.5%

* Significantly greater mortality than the negative control (Fisher's exact test, $p<0.05$).

Table 3.6. Some predicted target genes of miR-34 in *A. aegypti*.

Gene Symbol	Function
AAEL011878	Glucose transmembrane activity
AAEL010216	Transmembrane transport
AAEL006265	Neurogenesis
AAEL001477	Regulation of cell migration
AAEL001711	Developmental process
AAEL006778	Developmental process
AAEL005321	Developmental process

Like the dsRNA injection experiment, better survival of the mosquitoes was observed if adults were injected rather than pupae. Newly eclosed male mosquitoes were injected with 40 µM antisense oligonucleotide, and the surviving males were then mated to virgin females. The fecundity of the oligonucleotide-injected males was significantly reduced (Table.3.7), suggesting that miR-34 in *A. aegypti* is essential for testis development and/or spermatogenesis.

Table 3.7. Fecundity data obtained from mated antisense oligonucleotide injected males with uninjected females.

Treatment	# male adults injected	# blood-fed females	Total eggs hatched	# viable eggs/mated female
Buffer (control)	26	14	335	23.93±2.6
Aamir34m	32	14	15	1.07±0.4*****

***** indicates significant difference in number of viable eggs per mated females relative to the negative control (Fisher's exact test, $p<0.05$).

QRT-PCR was performed on 6 individual adult males that had been injected to determine the efficiency of knockdown by *aae/014067* dsRNA and Aamir34 antisense oligonucleotide (Fig 3.8). The expression level of *aae/014067* and aae-miR-34 revealed a significant knockdown, as ~ 87% knockdown of *aae/014067* and ~83% knockdown of *aae-miR-34* related to the negative controls.

Fig.3.8

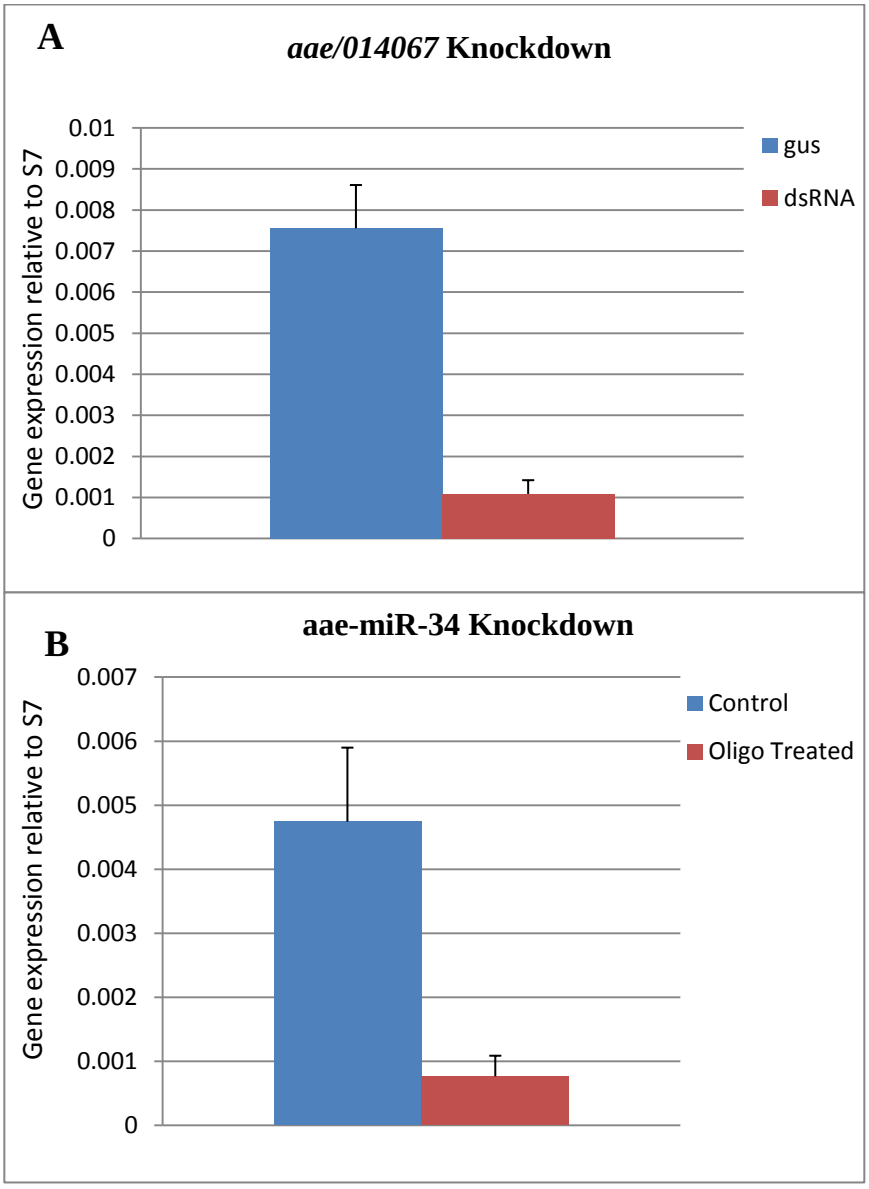


Figure 3.8. Expression levels of A) *aae/014067* and B) aae-miR-34 relative to ribosomal protein gene *S7*. Changes in *aae/014067* and aae-miR-34 expression in males injected with *aae/014067* dsRNA and *Aamir34m* oligonucleotide antisense compared to negative controls. The values represent the means and standard errors for the experiment performed in triplicate. All values were significantly different from one another (student t-test; $p<0.05$).

4. Discussion

Insects' genomes contain hundreds of miRNAs, which are involved in nearly every biological process, but little is known about the functions of miRNAs in testis development and spermatogenesis (Lucas and Raikhel, 2013). In this study, subsets of miRNAs that share homology with mammalian testis miRNAs were examined at both the tissue and developmental stage expression levels in *A. aegypti* and *D. melanogaster*. Some of the miRNAs examined showed high expression levels in the testis, relative to other tissues, suggesting that these miRNAs have potential roles in testicular development and/or spermatogenesis in both insects. This study also showed that one particular miRNA, miR-34, targets genes that either have functions in spermatogenesis or have a testis-specific expression pattern, which suggests that this miRNA may have critical roles in regulating male fertility in both insects.

A loss-of-function technique was then used to gain more insight into the function of miR-34 and one of its target genes, *aae/014067*, in *A. aegypti* male fertility. DsRNA and *Aamir34m* antisense oligonucleotides injections successfully knocked down expression of *aae/014067* and miR-34, respectively, resulting in significant reductions in mosquito male fecundity. These intriguing results suggest that both *aae/014067* and miR-34 are associated with *A. aegypti* male fertility and the disruption of their normal expression could render mosquitoes sterile.

4.1. Conservation of microRNAs

As new genome information for different species becomes available, comparative analyses will provide us with more tools to study the relationship of genome structure and

function across different biological species. *D. melanogaster* is one of the most studied organisms in biological research, particularly in genetics and developmental biology, due to its long history as a model species, but also because it was one of the first metazoans to have its genome fully sequenced and annotated (Severson *et al*., 2004). Despite an estimated 250 million year evolutionary divergence between *D. melanogaster* and *A. aegypti,* the *D. melanogaster* genome shares extensive homology with the *A. aegypti* genome (Severson *et al*., 2004; Nene *et al*., 2007), and hence the *D. melanogaster* genome was used in this study as a guide to identify miRNA homologues and target genes functions in *A. aegypti*. The miRNAs that were examined in this study were, in fact, 95-100% conserved in these two species.

Although closely related species are more likely to share thousands of conserved genes, distantly related species also share many conserved genes (Behura *et al*., 2011). For example, comparing the vinegar fly genome with the human genome revealed that about 60% of genes are conserved between these flies and humans (Rubin *et al*., 2000). Several studies have shown that the mature sequences of many miRNAs are conserved among different organisms, and these conserved miRNAs are likely to regulate biological functions common between invertebrates and vertebrates (Lagos-Quintana *et al* ., 2001 ; Bartel 2004; Lee *et al*., 2007; Ibáñez-Ventoso *et al* ., 2008). For example, over half of the *C. elegans* miRNAs share sequence homology with miRNAs expressed in both flies and humans (Ibáñez-Ventoso *et al*., 2008). This conversation across species suggests that identification of the role of a miRNA in one species may well inform the biological function of the homologous miRNA in other species (Ibáñez-Ventoso *et al*., 2008). For example, the *let-7* miRNA represses the human and mouse RAS oncogene expression, as

well as expression of the *C. elegans let-60* gene, which is the nematode's ortholog to human RAS (Jonson *et al*., 2005; Esqela-Kerscher *et al*., 2008). Nevertheless, conservation in miRNA sequences across species doesn't necessarily imply that the miRNAs share the same biological function. For instance, miR-1 displays significant diversity in its interaction and function among different species. In *Drosophila*, miR-1 contains one variant nucleotide and one extra nucleotide at the 3` end compared to the mammalian miR-1. In mammals, this miRNA has been shown to be involved in cardiac differentiation by targeting mRNA transcripts of the transcription factor Hand-2, while in *Drosophila*, miR-1 targets Delta transcripts, which encode a Notch ligand, and shows no specificity for transcripts of the Hand-2 *Drosophila* ortholog (Kwon *et al*., 2005; Zhao *et al*., 2005). Despite the high level of sequence identity of the miRNAs examined in *D. melanogaster* and *A. aegypti* in this current study, it is clear that such conservation does not imply conserved function in the two insects, and hence, it was important to examine where and when during development they are expressed, and to confirm whether they could actually bind to the same target transcripts.

4.2. Tissue specificity and developmental stages specificity of microRNAs

Several miRNAs in animals exhibit tissue-specific or developmental stage-specific expression, indicating that they could play important roles in different biological processes (Lagos-Quintana *et al*., 2001; Lee and Ambros 2001). Knowledge of tissue-specific and developmental-specific expression patterns of miRNAs can provide insight into their biological functions (Aboobaker *et al.*, 2005). For example, miR-375 is specifically expressed in mouse pancreatic islet cells, and was shown to be involved in regulating insulin secretion (Poy *et al*., 2004). *Drosophila* miR-1 is found exclusively in

muscle, where it regulates muscle physiology (Sokol and Ambros, 2005). Furthermore, miR-3 is expressed only during *Drosophila* embryogenesis and not at later developmental stages (Lagos-Quintana *et al*., 2001).

There are only a few reports on miRNAs' expression in the testis during insect spermatogenesis (Aravin et al., 2003; Skalsky *et al*, 2010; Cuevas *et al.*, 2011), but the functions of these miRNAs are not well-defined and the transcripts that they target have not been identified. In mammals, only a few testis miRNAs have been identified to date, but as they appear to be testis-specific or testis-enriched, they are suspected of serving a role in testis specification and cell lineage identification (Ro *et al*., 2007; Hossain *et al*., 2012). In this study, five presumed homologues of mammalian testis miRNAs were identified within the genomes of *A. aegypti* and *D. melanogaster*. An examination of their developmental and tissue expression confirmed that all miRNAs examined were expressed in the testis of both insects, and some of them, such as miR-34 in the mosquito and miR-219 in *D. melanogaster*, were highly expressed in the testis relative to the rest of the body. In mammals, miR-34 also displays high expression in the testis (Lagos-Quintana *et al*., 2002; Yan *et al*., 2009), which suggests that miR-34 may serve some conserved functions in vertebrates and invertebrates associated with testis development or spermatogenesis.

Interestingly, miR-9 showed exclusive expression in the testis in *A. aegypti*, while in *D. melanogaster*, it is highly expressed in testis, but is expressed in other tissues as well. This differential expression in the two insects is not unexpected, as miR-9, despite being highly conserved in many different species, has previously been observed to demonstrate strikingly different expression patterns across species (Yura-Aydemir *et al*.,

2011). For example, miR-9 in vertebrates is expressed largely in the nervous system, but in *Drosophila,* it is expressed predominantly in the imaginal discs that form the wings (Li *et al*., 2006; Yuva-Aydemir *et al*., 2011). The other miRNAs examined in this study did not show exclusive expression in the testis, but were expressed in other tissues, regulating either a variety of non-testis genes or genes expressed in both the testis and other tissues.

The developmental stage-expression of the miRNAs examined in this study also showed some differences and similarities between the two insects. In the mosquito, miR-124 was expressed primarily in pupae and adults, but it was expressed predominantly in *Drosophila* pupae. This finding suggests that the timing of expression of miR-124 is not strictly conserved between *Aedes* and *Drosophila,* and may reflect important developmental differences in the two insect species during the pupa to adult transition (Ason *et al*., 2006).

In contrast to miR-124 and its different developmental expression, miR-34 was predominantly expressed in adult males, relative to any other developmental stages, in both *Aedes* and *Drosophila,* which may indicate conserved function in the two flies. While each of the miRNAs are likely relevant to the development or function of the testis, the high expression of miR-34 in testis and adult males in both *A. aegypti* and *D. melanogaster,* along with the fact that this miRNA has documented roles in mammalian germ cell and gonadal somatic cells (Bohallier *et al*., 2010; *Bao et al*., 2012; Hossain *et al*., 2012), prompted a more in-depth analysis of this microRNA for the remainder of the study.

4.3. MicroRNA-target interactions

Identification of genes targeted by miRNAs is an essential step toward understanding the role of miRNAs in gene regulatory networks. In this study, three putative target genes for miR-34 were selected, based on testis or spermatogenesis functions and testis enhanced expression. One of the predicted targets of miR-34 was the transcript encoding porin. Porin is a small integral membrane protein present in the mitochondrial outer membrane; it is involved in the regulation of metabolite flux between the cytosolic and mitochondrial compartments (Graham *et al*., 2010). This gene exhibits the greatest homology to mammalian voltage-dependent anion channels (VDACs) and is ubiquitously expressed in all parts of the body in *Drosophila* (Park *et al*., 2010). Porin mutants in *Drosophila* display a variety of phenotypes, including lethality, defects of mitochondrial respiration, abnormal muscle mitochondrial morphology, synaptic dysfunction, and male infertility (Graham *et al*., 2010). The other two predicted targets of miR-34, *dme/CG8292* and *aae/014067*, have no known functions, but the fact that they were described as testis-specific and they were predicted to bind miR-34 also made them interesting candidate genes worthy of further examination.

As a part of the effort to understand the relationship between miRNAs and their targets, computational algorithms have been developed that are based on many features such as thermodynamic stability and the degree of hybridization between the two RNA molecules (reviewed in Ritchie *et al*., 2013). Although these computational algorithms are merely predictions and have not been functionally validated, they can provide valuable clues for potential targets. Two computational algorithms were used in this study to identify the aforementioned putative target genes for miR-34 in *A. aegypti* and *D.*

melanogaster. MicroInspector allows users to identify potential miRNAs that bind to specific mRNA sequence depending on free energy values (Rusinov *et al*., 2005) while TargetScan searches for conserved seed pairing regions in the 3`UTR alignments among different species (Yue *et al*., 2009). Because these algorithms used different features to make their predictions, they could identify different targets (Yue *et al*., 2009). For example, Porin in *Drosophila* was predicted as potential target for miR-34 in TargetScan but not in MicroInspector. Moreover, *dme/CG8292* was a predicted target for miR-34 in MicroInspector but not in TargetScan. Experimental examination of the binding activity of the microRNAs was therefore required to assess which algorithm was a more accurate predictor of a microRNA's function. The cell-based assays that were conducted in this study provided evidence that miR-34 can bind to the 3'UTR sequences of porin transcripts in *D. melanogaster* and *A. aegypti* as well as the 3' UTRs of *dme/CG8292* and *aae/014067* transcripts. Evidently, despite only one algorithm predicting each target, miR-34 appears to bind to the 3`UTRs and inhibit the expression of the three target genes examined. With only a few genes to consider from this study, it is not possible to assess which algorithm will be a better predictor of any one particular miRNA's function. Further refinements of the algorithms will be required, and cell-based assays could assist in determining the true binding potential of microRNAs to their target mRNAs. It is important to recognize that these cell-based assays only provide supportive evidence that miR-34 could down-regulate expression of these target genes; they do not provide definitive proof that the miRNA is regulating those genes in the intact insect. Further *in vivo* analyses in the intact insect were needed to make definitive conclusions on the miRNA's function in the insect's tissues.

4.4. Functional analysis of *aae/014067*

RNA interference (RNAi) is a powerful method for determining the role of a specific gene. Following delivery of dsRNA with sequence complementary to a targeted gene of interest, RNAi will ensue, where a drastic decrease in the expression of a targeted gene is observed. In recent years, RNAi has proven to be an effective strategy for inhibiting gene function and observing loss-of-expression phenotypes in many insect orders including Diptera, Lepidoptera, Coleptera, and Hymenoptera (reviewed in Yu *et al*., 2013).

In this study, mosquitoes were injected at both pupal and adult stages with dsRNA to study the physiological role of *aae/014067*, as this gene has no known function. Curiously, injections of the pupae with *aae/014067*-dsRNA caused significant mortalities relative to the negative controls, which were injected with *gus*-dsRNA. Based on transcriptomic data in FlyBase (http://flybase.org/), *aae/014067* gene expression is limited to the testis, and hence, it was unexpected that the dsRNA injection would be lethal to pupae. One possible explanation for the high mortality following *aae/014067*-dsRNA injections is that, contrary to what the transcriptomic database dictates, this particular gene is in fact expressed in other tissues and is critical for the development of the pupa. Alternatively, the high mortality in mosquito pupae could be the consequence of off-target effects of the injected dsRNA. Although BLAST searches indicated no 19-21 nucleotide homology match of the *aae/014067*-dsRNA to any genes in the *A. aegypti* genome, it's possible that the siRNAs derived from the processed dsRNA could act as miRNAs and repress an essential pupal developmental gene's expression via imperfect complementarity (Doench *et al*., 2003).

In contrast to the poor survival of the dsRNA-injected pupae, higher survivorship was observed in adult mosquitoes injected with *aae/014067* dsRNA, with only about 10% of the adults dying, which was comparable to the mortality observed in the negative controls. If off-target effects of the dsRNA were the cause of the pupal mortality, no such off-target effects were observed in the adults, presumably because the off-target genes were not expressed in adults or that knock down of the genes in the adults did not affect survival. QRT-PCR confirmed that the dsRNA induced significant knockdown of the target gene transcripts, and reduced the fecundity of the injected males by approximately 95% relative to the negative control mosquitoes that were injected with the *gus* dsRNA. While the actual role of this gene is still not fully resolved, it is appears to be associated with *A. aegypti* male fertility, suggesting that *aae/014067* is a promising candidate gene for sterilizing mosquitoes for an SIT control program.

4.5. MicroR-34 is crucial for mosquito male fertility

A relatively new approach to inducing miRNA loss-of-function is to use chemically modified antisense oligonucleotides, which bind to the mature miRNA, leading to functional inhibition of the miRNA (Torres *et al*., 2012; Stenvang *et al*., 2012). These antisense oligonucleotides are single-stranded RNA-based inhibitors that are chemically modified to improve their activity and to increase their stability (Torres *et al*., 2012). MiRNA gene knockdowns have been used previously to gain understanding of miRNAs functions in *C. elegans* and *Drosophila*, and have also been reported for assessing functions of miRNAs in the mouse (Abbott *et al*., 2005; Williams *et al*., 2009; Mu *et al*., 2009; Rooij *et al*., 2009). Here, an antisense oligonucleotide, which is a 2′-O-

Me/DNA phosphorothioate chimera oligonucleotide, was used to investigate the role of miR-34 in *A. aegypti* male fertility.

Mosquito pupae injected with different dosages of the *Aamir34m* antisense oligonucleotides showed high mortality compared to the negative control. This result is not surprising, as antisense oligonucleotides can affect RNA species other than the intended miRNA target (Stenvang *et al*., 2012). Since animal miRNAs bind with partial complementarity to their targets, it is possible that the *Aamir34m* antisense oligonucleotides could bind to some of the same genes that are predicted to bind to miR-34, some of which could be genes essential for pupal developmental; loss of function of many of these could prove lethal to the developing pupa. Alternatively, the *Aamir34m* antisense oligonucleotide could target other miRNAs that are essential for development, given that many miRNAs possess similar or even identical seed sequences (Bao *et al*., 2012).

Like the dsRNA injection experiment, better survival of the mosquitoes was observed if adults were injected with the antisense oligonucleotide rather than pupae. Newly eclosed male mosquitoes were injected with 40 µM antisense oligonucleotide and qRT-PCR revealed a significant knockdown of miR-34 related to the negative controls. The surviving males were then mated to virgin females to assess the role of miR-34 in mosquito fertility. The fecundity of the oligonucleotide-injected males was significantly reduced, suggesting that miR-34 in *A. aegypti* is essential for testis development and/or spermatogenesis. This not surprising, as many previous studies demonstrated that miR-34 plays critical role in male germ cell development (Bouhallier *et al*., 2010; Bao *et al*., 2012; Liang *et al*., 2012). Nevertheless, it would be interesting to examine the testis

morphology of mosquitoes injected with *Aamir34m* antisense oligonucleotides, as this could provide a better understanding about the role of miR-34 in *A. aegypti* fertility. It would also be of interest to determine whether the antisense oligonucleotide has an impact on the levels of proteins encoded by the RNAs affected by miR-34; presumably, the *aae/014067*-encoded protein levels would increase with the application of the microRNA-inhibiting oligonucleotide, but confirmation of this would require the development of antibodies to detect such changes.

4.6. Future Directions and Conclusions

In this study, several microRNAs showing a testis-specific or -enhanced expression were identified, and using computer algorithms, several candidate target genes were identified. The accuracy of these predictive algorithms has not been adequately determined, but using cell based-reporter gene assays, it will be possible to assess the predictions, and ultimately improve our ability to determine a microRNA's function. We have much to understand about how many genes a single miRNA typically regulates, and how that regulation actually impacts on the cell's and/or organism's phenotype.

While the cell-based assays are informative about the mRNA binding capabilities of microRNA, they do not inform us of the *in vivo* functions of the microRNAs. The use of microRNA-inhibiting oligonucleotides can help define a microRNA's function, but this method can be complicated by the multiplicity of impacts that each microRNA has on any single cell, and even more confounding when examining body-wide impacts. With continued improvements in high-throughput RNA sequencing and reduction in costs, it

will soon be possible to examine the many impacts following miRNA inhibition, and thereby gain a more complete understanding of miRNA functions.

The analysis of testis microRNAs in the two insects also helped identify a few target genes that are relevant to testis development and/or function. Disruption of the expression of either the microRNA itself or of one of its target genes was shown to impact the fertility of the mosquito. Using similar screens to identify genes essential for male fertility, it will be possible to develop new technologies to control pest insects. SIT is a method of controlling insects by producing large numbers of sterilized males, which are released into an environment to compete with wild males and reduce the population. The method has not been widely used on many species as it has proven challenging to find effective methods of sterilizing the insects without adversely affecting their mating abilities (William *et al*., 2011). Using RNAi screens such as the one used in this study will help identify which genes could serve as targets in the development of RNAi-mediated insect sterilization method; however, an added challenge will be to find target genes that don't affect the male's willingness to mate. Alternatively, identification of relevant microRNAs that regulate fertility could be targeted – the challenge is to find effective methods of delivering microRNA inhibitory compounds to the insects.

In conclusion, this study provides new insights into the role of some microRNAs relevant to testis function in two dipteran insects. Although there is much more to understand about the complexity of microRNAs and their roles in regulating cellular functions, this study also provides some possible new directions for the development of novel methods of controlling one of our most serious disease-vectoring mosquitoes.

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