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MicroRNAs are differentially abundant during *Aedes albopictus* diapause maintenance but not diapause induction

Zachary A. Batz[*], **Allison C. Goff**, and **Peter A. Armbruster**

Department of Biology, Georgetown University, 37th and O Streets NW, Washington, DC, USA 20057

Abstract

Diapause is a programmed dormancy that allows organisms to tolerate predictable periods of unfavorable conditions by temporarily halting development and reducing metabolism. Diapause is widespread among insects and is crucial for allowing organisms to coordinate their growth and reproduction with favorable environmental conditions. While the adaptive significance of diapause is well-understood, the molecular mechanisms underpinning diapause remain unresolved. We performed high-throughput sequencing to investigate the role of microRNAs in the diapause of the Asian tiger mosquito, *Aedes albopictus.* We first investigate miRNAs in diapause induction by characterizing maternally-provisioned miRNAs in mature oocytes of *Ae. albopictus* under diapause-inducing and diapause-averting conditions. Second, we investigate miRNAs in diapause maintenance by characterizing miRNAs in diapause and non-diapause pharate larvae. We identified 162 miRNAs, 152 previously known and 10 putatively novel. We identified no differentially abundant miRNAs in mature oocytes and seven differentially abundant miRNAs in pharate larvae. The predicted targets of differentially abundant miRNAs include genes affecting several processes related to diapause maintenance including ecdysone regulation, immune response, lipid metabolism, and regulation of development. Our results suggest that *Ae. albopictus* does not maternally-provision a unique set of miRNAs during diapause induction but miRNAs are a component of diapause maintenance in this species.

Keywords

diapause maintenance; diapause induction; microRNA (miRNA) regulation; *Aedes albopictus*

Introduction

All temperate insects face seasonally recurring environmental stress. For these species, overwinter survival requires a behavioral response, such as migrating to a warmer climate, and/or a physiological adaptation like diapause (Saunders, 2009). Diapause is an alternative life-history strategy characterized by a period of developmental arrest in response to a token

[*]Author for correspondence – zab7@georgetown.edu.

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cue (i.e., photoperiod) detected in advance of unfavorable conditions. Diapause involves a progression through four ecophysiological stages (Koštál, 2006). In the first stage, induction, the organism perceives the token cue(s) that initiate the diapause program. In the second stage, preparation, the organism undergoes physiological and developmental changes in preparation for entry into diapause. The third stage, maintenance, is characterized by reduced metabolism, developmental arrest, and a suite of physiological modifications to facilitate survival including increased stress tolerance, elevated immunity, and downregulation of the cell cycle (Goto *et al.*, 2001; Denlinger, 2002; King & MacRae, 2015; Denlinger & Armbruster, 2016). Finally, during the fourth stage, termination, the organism becomes competent to resume growth and development. Diapause enables insects to maximize their fitness by synchronizing growth and reproduction with periods of favorable environmental conditions (Tauber *et al.*, 1986). While the adaptive significance of diapause is well-established, the underlying molecular mechanisms regulating this complex phenotype are less clear.

The Asian tiger mosquito, *Aedes albopictus* (Skuse), is an emerging model for investigating the molecular underpinnings of diapause. *Ae. albopictus* is a highly invasive species and diapause has facilitated its establishment throughout temperate regions (Benedict *et al.,* 2007; Urbanski *et al.,* 2012). In *Ae. albopictus*, diapause is under maternal control; pupal and adult females detect short (autumnal) photoperiods as a token cue and respond by producing diapause offspring which overwinter as pharate larvae within the chorion of the egg (Mori & Oda, 1981; Denlinger & Armbruster, 2016). However, the mechanisms by which the mother induces diapause in offspring remain elusive. Diapause eggs are more cold tolerant and desiccation resistant than non-diapause eggs (Sota & Mogi, 1992; Hanson & Craig, 1994, 1995; Urbanski *et al.,* 2010). Also, diapause eggs are larger and contain ~40% more surface hydrocarbons and ~30% more total lipids than non-diapause eggs (Urbanski *et al.,* 2010; Reynolds *et al.,* 2012). In addition to these physiological changes, transcriptome sequencing has identified thousands of coding genes that are differentially expressed throughout the four stages of diapause (Poelchau *et al.,* 2011, 2013a,b; Huang *et al.*, 2015).

Small, non-coding RNAs (sncRNAs) have been shown to regulate a wide range of developmental and physiological processes (Hussain *et al.*, 2016). Regulatory sncRNAs are divided into three main classes according to their biogenesis pathway: small-interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and microRNAs (miRNAs) (Lucas *et al.*, 2013). siRNAs are ~21nt long double-stranded RNA molecules (dsRNA) which originate either endogenously from sequences in the genome or exogenously from foreign dsRNA (Hussain *et al.,* 2016). piRNAs are ~24-31nt long and canonically guide cleavage of mobilized transposable elements in germline cells (Ishizu *et al.*, 2012). Additionally, piRNAs can regulate the expression of protein coding genes (Iwasaki *et al.*, 2015). miRNAs are ~22nt long and synthesized in pairs from a hairpin precursor (pre-miRNA) (Bartel, 2004). Members of a miRNA pair are denoted with either a -3p or -5p suffix corresponding to the arm of the hairpin precursor from which it was synthesized (Desvignes *et al.*, 2015). miRNAs post-transcriptionally regulate gene expression by guiding an *Argonaute* protein complex to silence target mRNAs containing perfect or near-perfect complementarity to a 7nt seed region in the miRNA (Bartel, 2009). The short seed sequence allows for a single

miRNA to target and affect the translation of dozens or even hundreds of genes (Lewis *et al.*, 2005; Baek *et al.*, 2008).

Several studies have implicated miRNAs in the regulation of diapause and diapause-associated physiological traits. Genes encoding proteins involved in miRNA biogenesis and function are differentially expressed during diapause maintenance in the flesh fly, *Sarcophaga bullata* (Reynolds *et al.,* 2013). Specifically, *Loquacious*, encoding a protein necessary for miRNA maturation, and *Argonaute-1,* encoding a protein in the miRNA-induced silencing complex, are upregulated in diapause pupae of *S. bullata* (Reynolds *et al.*, 2013). Furthermore, ten miRNAs were found to be differentially abundant during pupal diapause in *S. bullata* and subsequent *in silico* target prediction suggests these miRNAs could regulate development, stress tolerance, and metabolism (Reynolds *et al.,* 2017). miRNAs also contribute to maintenance and termination of the dauer arrest in *Caenorhabditis elegans*, a diapause-like state in worms (Karp *et al.*, 2011; Karp & Ambros, 2012). miRNAs are also known to regulate numerous pathways associated with diapause physiology such as metabolism, development, cell signaling, growth, apoptosis, cell division, and immunity (Huang *et al.*, 2011; Asgari, 2012; Ebert & Sharp, 2012; Biggar & Storey, 2015; Lucas *et al.*, 2015). Finally, miRNAs are known to be maternally provisioned in *Drosophila melanogaster* eggs (Votruba, 2011; Soni *et al.*, 2013). Given the differential expression of genes involved in miRNA function and biogenesis, the role of miRNAs in regulation of diapause-related pathways, and the potential for miRNA transgenerational signaling, we hypothesized that miRNAs could be an important component of diapause regulation in *Ae. albopictus*.

In this study, we utilized next-generation sequencing to identify novel miRNAs in *Ae. albopictus.* We then analyzed the next-generation sequencing data to quantify and compare the abundance of miRNAs present in mature oocytes of adult females reared under diapause-averting and diapause-inducing photoperiod as well as miRNAs of pharate larvae in early diapause maintenance relative to developmentally-matched non-diapause pharate larvae. Finally, we predicted target sites for differentially abundant miRNAs and identified diapause-related functions of miRNA target genes.

Results

Diapause incidence

Diapause incidence was measured in replicates from both the mature oocyte (induction) and pharate larvae (maintenance) experiments (Fig. 1) to confirm that short-day (SD) photoperiod conditions had induced diapause and that long-day (LD) photoperiod conditions had induced non-diapause. In the mature oocyte experiment, diapause incidence was 96.7–98.4% in two SD photoperiod replicates and 16.1–18.0% in two LD photoperiod replicates. In the pharate larvae experiment, diapause incidence was 93.5–98.9% in four SD photoperiod replicates and 2.6–12.1% in four LD photoperiod replicates (Table S1).

RNA libraries and read mapping

All reads generated in this project are available in the NCBI Short Read Archive with the accession numbers SRR5168334-SRR5168339 (mature oocytes) and SRR5168326-SRR5168333 (pharate larvae). Six mature oocyte libraries and eight pharate larvae libraries were sequenced, cleaned, and size sorted producing a total of 12.9 million cleaned reads (Table 1). The read lengths obtained in these two experiments were significantly different (Mann-Whitney $U = 1.23 \times 10^5$, $p < 0.001$; Table S2). Mature miRNA length reads (20–22nt) comprised 17.7% of the mature oocyte libraries compared to 46.7% in the pharate larvae libraries. Mature piRNA length reads (24-31nt) comprised 58.4% of the mature oocyte libraries and just 15.9% of the pharate larvae libraries.

To identify putatively novel miRNAs that have not previously been annotated for *Ae. albopictus*, reads from all 14 libraries across both experiments were combined, mapped to the *Ae. albopictus* genome, and scored with miRDeep2. Ten putatively novel miRNA precursors had miRDeep2 scores >4, hairpin loops in the known size range for *Ae. albopictus*, presented at least ten mature reads for each arm, and appeared in at least two libraries (Table S3). Eight of the predicted novel miRNA precursors were identified in both the mature oocyte and pharate larvae experiments. No putative novel miRNA precursors were exclusively identified in the mature oocyte libraries and two putative novel miRNA precursors were exclusively identified in the pharate larvae libraries. One novel miRNA precursor appeared only in diapause libraries and no novel miRNA precursors appeared in only non-diapause libraries (Table S3). Each precursor miRNA produces two distinct mature miRNAs; these mature miRNAs were searched in miRBase (Kozomara & Griffiths-Jones, 2014) to identify Dipteran orthologs. Five of the predicted novel mature miRNAs have seed regions identical to known Dipteran miRNAs, four in *Aedes aegypti* and one in *D. melanogaster*. All 20 predicted novel mature miRNA sequences were added to the set of previously annotated *Ae. albopictus* miRNAs (Skalsky *et al.*, 2010; *Gu et al.*, 2013) to produce a final reference set of 257 mature miRNAs (Table S4).

Next, each of the libraries was mapped to the *Ae. albopictus* genome and read counts were obtained for each miRNA in the reference set. For the mature oocytes, 48.3% of cleaned reads aligned to the genome and of these aligned reads, 19.1% mapped to miRNAs in the reference set. For the pharate larvae, 64.9% of cleaned reads aligned to the genome and of these aligned reads, 67.6% mapped to miRNAs in the reference set (Table 1). In total, 162 mature miRNAs were identified: 89 in both mature oocytes and pharate larvae, 12 only in the mature oocytes, and 61 only in the pharate larvae.

MicroRNA analyses

The distance between the miRNA expression patterns of each library was visualized in an MDS plot (Fig. S1). Variation within each treatment group (diapause, non-diapause) and stage (mature oocytes, pharate larvae) was estimated by the biological coefficient of variation (BCV) in *edgeR*. BCV was higher in the mature oocyte libraries (diapause: 0.796, non-diapause: 0.351) than in the pharate larvae libraries (diapause: 0.233, non-diapause: 0.182).

Next, miRNAs were tested for differential abundance between diapause and non-diapause conditions for oocytes and pharate larvae. No miRNAs had significant differences in abundance between diapause and non-diapause mature oocytes (Fig. 2a). However, three miRNAs had absolute $\log_2$-fold changes >2 (*aal-miR-1890-1-3p*, *aal-miR-957-3p*, and *aal-miR-92a-5p*).

Seven miRNAs were differentially abundant between diapause and non-diapause conditions in pharate larvae (Fig. 2b). Four miRNAs were over-represented and three miRNAs were under-represented under diapause relative to non-diapause conditions. The absolute values of $\log_2$-fold changes ranged from 0.41–1.58 (Table 2). All seven differentially abundant miRNAs were from the set previously annotated in *Ae. albopictus* (Skalsky *et al.*, 2010; Gu *et al.*, 2013). Fold change values measured during follow-up quantitative reverse-transcription PCR (qRT-PCR) experiments were strongly correlated to those obtained in the RNA-Seq analysis (r = 0.93, Table S5, Figure S2).

MicroRNA target prediction

miRNA target prediction was used to characterize the potential downstream effects of regulation by differentially abundant miRNAs at the pharate larvae stage (early diapause) using three target prediction algorithms (Fig. 3a). The first algorithm, TargetScan, predicted 546 target sites in 412 3′ UTRs. RNAHybrid, predicted 801 target sites across 780 3′ UTRs and miRanda predicted 444 targets in 393 3′ UTRs. In total, 147 3′UTRs were predicted to contain miRNA target sites by at least two algorithms (Fig. 3a). Three of these 3′UTRs were predicted to be targeted by two miRNAs identified as differentially abundant and seven of 3′UTRs were predicted to contain more than one target site for a single differentially abundant miRNA (Table S6).

GO-Slim2 annotations for genes containing predicted target sites were clustered by semantic similarity and sized according to frequency among the predicted target set (Fig. 3b). Changes in metabolism are a key feature of diapause and GO terms related to metabolism appear frequently within the set especially lipid, nucleobase, and protein metabolism.

Discussion

Diapause is a programmed dormancy that allows insects to anticipate and persist through periods of poor environmental conditions (Koštál, 2006). In the photoperiodically-induced diapause of *Ae. albopictus,* the pupal and adult female is photosensitive but developmental arrest occurs in the pharate larvae; the nature of the trans-generational signal is unknown (Denlinger & Armbruster, 2016). miRNAs can be maternally provisioned (Votruba, 2011; Soni *et al.*, 2013) suggesting they may play a role in this trans-generational signaling. In *S. bullata,* genes encoding proteins involved in miRNA biogenesis and function undergo coordinated changes in expression during diapause preparation and initiation (Reynolds *et al.*, 2013) and miRNAs are differentially abundant during diapause maintenance (Reynolds *et al.*, 2017). Thus, we hypothesized that maternally-provisioned miRNAs in mature oocytes may be a component of the transgenerational diapause signal in *Ae. albopictus*. Furthermore, diapause maintenance requires coordinated regulation of diverse physiological processes including: developmental arrest, reduced metabolism, and increased immunity and stress

tolerance (Denlinger, 2002). Because an individual miRNA can alter the translation of dozens or hundreds of genes via post-transcriptional regulation (Baek *et al.*, 2008; Asgari, 2012; Lucas *et al.*, 2015), individual miRNAs have the potential to underlie broad regulatory changes like those observed in diapause. Accordingly, we further hypothesized that key elements of the *Ae. albopictus* diapause phenotype may be regulated by differential abundance of miRNAs in pharate larvae during developmental arrest.

Putative novel miRNAs

miRDeep2 analysis of mature oocytes and pharate larvae small RNA samples led to the prediction of ten novel miRNA precursors (Table S3). miRNAs are often expressed in a stage-specific manner (Li *et al.*, 2009; Gu *et al.*, 2013) and no previous study has characterized *Ae. albopictus* miRNAs from either mature oocytes or pharate larvae. Two novel miRNA precursors were unique to pharate larval stage (Table S3).

Mature Oocyte sncRNAs

We characterized maternally-provisioned miRNAs by sequencing small RNA from mature oocytes dissected from virgin females reared under SD (diapause-inducing) or LD (diapause-averting) photoperiod. Relatively few miRNA-length reads were recovered (17.7% of cleaned reads; Table S2). In total, 101 miRNAs were present across six mature oocyte replicates. Contrary to our expectations, none of these miRNAs were differentially abundant between SD and LD photoperiods (Fig. 2). This result implies that maternally-provisioned miRNAs do not significantly contribute to the maternal regulation of the egg diapause in *Ae. albopictus.*

Intriguingly, 58.4% of the reads from mature oocytes were 24-31nt long, the expected size range of piRNAs (Table S2). This result is consistent with evidence from *S. bullata* that genes involved in piRNA biogenesis and function are upregulated during the photosensitive stage prior to diapause (Reynolds *et al.*, 2013). Canonically, piRNAs are involved in transposon defense but recent work demonstrates that these molecules can also regulate the expression of protein coding genes (Iwasaki *et al.*, 2015). In *Ae. aegypti*, just 19% of putative piRNAs are predicted to target transposable elements suggesting that non-canonical piRNA functions like targeting of protein coding genes are prevalent (Arensburger *et al.*, 2011). Preliminary analysis of piRNAs in *Ae. albopictus* predicted that approximately 20% of piRNAs map to protein-coding genes (Liu *et al.*, 2016). Thus, it seems likely that protein-coding genes may be regulated, at least to some extent, by piRNAs in this species. However, the preliminary annotation of *Ae. albopictus* piRNAs is less robust than for miRNAs (Liu *et al.*, 2016), and thus we did not attempt to quantify differential abundance of piRNAs associated with diapause in this study.

Pharate Larvae miRNAs

We characterized miRNAs in early diapause maintenance by sequencing small RNAs from pharate larvae in either early diapause or non-diapause. In contrast to the low proportion of miRNAs in the mature oocytes, 46.7% of cleaned reads in the pharate larvae RNA samples were in the miRNA size range (Table S2) and 67.7% of genome-aligned reads mapped to reference miRNAs (Table 1). 150 miRNAs were identified across a total of eight diapause

and non-diapause replicates. Seven miRNAs were differentially abundant in early diapause pharate larvae; four were up-regulated and three were down-regulated (Fig. 2, Table 2). A recent study identified ten differentially abundant miRNAs during the pupal diapause of *S. bullata* (Reynolds, *et al.,* 2017) including *miR-1-3p*, one of the differentially abundant miRNAs identified in our study. In contrast to our results, *miR-1-3p* was more abundant in *S. bullata* during diapause relative to non-diapause, suggesting independent mechanisms of miRNA regulation of diapause in these two species. The seven differentially abundant miRNAs in the pharate larvae of *Ae. albopictus* have been implicated in processes associated with diapause including increased immunity, lipid accumulation and developmental regulation.

Differentially Abundant miRNAs in Pharate Larvae

miR-283-5p and *miR-1-3p* were both differentially expressed in pharate larvae and have been linked to immune response in *Ae. albopictus. miR-283-5p* was upregulated and *miR-1-3p* was absent in an *Ae. albopictus* cell line after exposure to Chikungunya virus (Shrinet *et al.* 2014). Similarly, in our study *miR-283-5p* was upregulated and *miR-1-3p* was downregulated in diapause pharate larvae. Enhanced immune function is thought to be an important component of diapause (Chernysh *et al.*, 2000; Ragland *et al.*, 2010), and we hypothesize that differential abundance of these two miRNAs may contribute to increased immunity during diapause.

miR-14-5p was downregulated during diapause in pharate larvae and has previously been linked to regulation of lipid metabolism. Knockout of the *mir-14* precursor in *D. melanogaster* reduces lipid metabolism and increases lipid accumulation in the form of triglycerides and diglycerides (Xu *et al.*, 2003). Previous work in *Ae. albopictus* found that diapause eggs have approximately 30% more lipids than non-diapause pharate eggs, due at least in part to upregulation of triglyceride storage and downregulation of lipid catabolism during diapause preparation (Reynolds *et al.*, 2012). Thus, the diapause-associated down-regulation of *miR-14-5p* in our data is consistent with that evidence suggesting that differential abundance of this miRNA could contribute to the accumulation of lipids.

Developmental arrest is a hallmark of diapause and thought to be regulated in most insects by some combination of two hormones: juvenile hormone (JH) and ecdysone (Denlinger *et al.*, 2012). For example, high levels of ecdysone initiate and maintain the pharate larval diapause of *Lymantria dispar* (Lee & Denlinger, 1997). In contrast, low levels of ecdysone maintain egg diapause in *Locusta migratoria* (Tawfik *et al.*, 2002), larval diapause in *Omphisa fuscidentalis* (Singtripop *et al.*, 1999), and pupal diapause in *Mamestra brassicae* (Endo *et al.*, 1997). In *Ae. albopictus*, ecdysone signaling genes are differentially expressed in diapause-induced mature oocytes and embryos (Poelchau *et al.*, 2011, 2013a). During diapause maintenance, genes related to JH binding and metabolism, but not ecdysone, are differentially expressed (Poelchau *et al.*, 2013b). These previous findings implicate differential ecdysone signaling in *Ae. albopictus* diapause preparation but not diapause maintenance.

Our study provides evidence that post-transcriptional regulation of ecdysone-related genes by miRNAs could be involved in early diapause maintenance in *Ae. albopictus* pharate

larvae. Two miRNAs linked to ecdysone regulation, *bantam-5p* and *miR-282-5p*, were overexpressed in diapause pharate larvae (Table 2). In *D. melanogaster*, *bantam* negatively regulates ecdysone production in the prothoracic gland and strong overexpression significantly extends time to pupation (Boulan *et al.*, 2013). *miR-282-5p* negatively regulates the *rutabaga* gene in *D. melanogaster* (Vilmos *et al.*, 2013). *Rutabaga* is most often associated with adult learning and memory functions (Livingstone *et al.*, 1984) but mutation of this gene has also been found to delay larval molts by limiting the production of active ecdysone (Venkatesh *et al.*, 2001). Together, these functional studies in *D. melanogaster* suggest that *bantam-5p* and *miR-282-5p* may negatively regulate ecdysone synthesis to suppress larval development.

Finally, *miR-2942-3p* and *miR-286b-3p* were differentially abundant in diapause pharate larvae and both have been linked to developmental transitions in *Ae. albopictus* (Puthiyakunnon *et al.*, 2013). Knockdown of *miR-2942-3p* in *Ae. albopictus* larvae significantly reduces the rate of eclosion from pupa to adult (Puthiyakunnon *et al.*, 2013). In our study, *miR-2942-3p* was downregulated in the diapause pharate larvae, consistent with the hypothesis that reduced levels of *miR-2942-3p* impede developmental transitions. Similarly, knockdown of *miR-286b-3p* in *Ae. albopictus* embryos delayed hatching and reduced hatching success (Puthiyakunnon *et al.*, 2013). Delayed hatching is a primary component of diapause in *Ae. albopictus,* therefore we expected *miR-286b-3p* to be downregulated in our diapause pharate larvae. In contrast to this expectation, *miR-286b-3p* was upregulated under diapause. In some cases, both overexpression and knockdown of a particular miRNA generate similar mutant phenotypes (e.g. Hansen *et al.*, 2013; Luo & Sehgal, 2012). Thus, one possible explanation is that an intermediate abundance of *miR-286b-3p* is required for normal hatching. The differential abundance of these two miRNAs related to developmental transitions further support the notion that differentially abundant miRNAs play a role in regulating the suppression of development during early diapause maintenance in *Ae. albopictus*.

miRNA Target Characterization

To characterize additional downstream regulatory effects, we identified putative gene targets for each of the seven differentially abundant miRNAs in pharate larvae. 147 genes were predicted to contain target sites for the differentially abundant miRNAs by at least two algorithms (Fig. 3a, Table S7). Among these targets, 58 genes were annotated with the GO-Slim2 term metabolism (Fig. 3b, Table S7). Of particular interest, four metabolism gene targets were annotated as components of lipid metabolism. Lipids are the primary energy source for most diapause insects (Hahn & Denlinger 2011) and altered lipid metabolism is a critical component of the diapause phenotype in *Ae. albopictus* (Reynolds *et al.*, 2012; Poelchau *et al.*, 2013b). One of the predicted miRNA target genes (AALF015949) is an ortholog of the *D. melanogaster* gene *Hormone-sensitive lipase* (*HSL*). *HSL* expression is induced by starvation and mobilizes triglycerides from intracellular lipid storage droplets for lipolysis (Bi *et al.*, 2012). miRNA targeting of the *HSL* gene could potentially help fine-tune starvation response during diapause to conserve lipids. Triglyceride lipases were previously found to be downregulated during *Ae. albopictus* diapause preparation (Reynolds *et al.*, 2012) and upregulated during early diapause maintenance (Poelchau *et al.*, 2013b). Our

results suggest that post-transcriptional targeting by miRNAs could provide another level of regulation for lipid metabolism in *Ae. albopictus* diapause.

We identified five putative target genes related to cell cycle regulation and apoptosis, two pathways typically downregulated during diapause (Hwang *et al.*, 2005; Koštál *et al.*, 2009; MacRae, 2010; Poupardin *et al.*, 2015). In *Ae. albopictus,* differential expression of genes regulating the cell cycle and apoptosis have been previously observed in diapause-destined mature oocytes and early in diapause preparation (Poelchau *et al.*, 2011, 2013a) but not during diapause maintenance (Poelchau *et al.*, 2013b). Thus, this study provides the first support for regulation of these pathways during the diapause maintenance of *Ae. albopictus.*

Conclusions

This study is the first to directly investigate changes in miRNA abundance as a mechanism of diapause regulation in *Ae. albopictus*. We found that maternally-provisioned miRNAs did not differ between diapause-induced and diapause-averting mature oocytes suggesting that miRNAs do not act as the transgenerational signal for diapause induction in *Ae. albopictus*. We found seven differentially abundant miRNAs in diapause pharate larvae which are implicated in a wide variety of diapause-associated traits including developmental arrest, stress tolerance, immunity, and shifts in metabolism. Collectively, these results suggest that miRNAs may play an important role in orchestrating physiological processes during early diapause maintenance of *Ae. albopictus*.

Experimental Procedures

Insect rearing and tissue preparation

An overview of the experimental design for this study is provided in Figure 1. For both experiments (mature oocytes = diapause induction, pharate larvae = diapause maintenance), a laboratory colony of *Ae. albopictus* from Manassas, Virginia, USA (38.6°N, 77.4°W) was reared as described previously (Armbruster & Hutchinson, 2002; Armbruster & Conn, 2006) and briefly summarized below.

Mature oocytes

Samples of mature oocyte tissue were generated by rearing F_7 *Ae. albopictus* larvae under LD photoperiod (16h light:8h dark) at 21°C, 80% humidity. Larvae were fed a near-optimal diet consisting of a slurry of dog food and brine shrimp (Armbruster & Hutchinson, 2002; Armbruster & Conn, 2006). Upon pupation, three replicate adult cages were established under SD photoperiod (8h light:16h dark) to induce diapause and three replicate adult cages were established under LD photoperiod to induce direct development (non-diapause). Each cage consisted of approximately 30 females. Cages were lined with a moist filter paper to help maintain humidity and females were provided organic raisins for sugar-feeding.

Virgin females were allowed to blood feed to repletion on a human host 5–8d post-eclosion. The Georgetown University Institutional Review Board (IRB) determined that mosquito blood feeding was not human research and thus did not require IRB approval. However, the blood feeding protocol was approved by the Georgetown University Occupational Health

and Safety Committee. Five days post-blood meal, females from each cage were briefly anesthetized with CO_2 and transferred to a 1.5mL tube. Tubes were snap frozen in liquid nitrogen and stored at -80°C. Mature oocytes, indicated by a clearly visible surface exochorion pattern (Clements, 1992), were dissected directly into RNA Later (Thermo-Fisher, Waltham, Massachusetts) from at least 13 females per cage and stored at 4°C overnight prior to RNA extraction (see below).

Diapause incidence

For both the mature oocyte and pharate larvae experiments, eggs from adult cages were collected to quantify the diapause incidence under SD and LD conditions. For the mature oocyte experiment, four mixed sex (50 male: 50 female) adult cages were established in parallel to the female-only cages used to produce mature oocyte tissue. Two cages were placed under SD photoperiod to induce diapause and two cages were placed under LD photoperiod to induce non-diapause. All females were allowed to blood feed to repletion 5–8d post-eclosion. Starting 4d post-blood meal, females were provided with a brown oviposition cup half-filled with deionized water and lined with unbleached paper towel. Eggs on the paper towel were collected after 24h, maintained for 48h, then gently air dried and stored at 21°C and 80% relative humidity in a sealed Tupperware container. For the pharate larvae experiment, diapause incidence was measured using approximately 100 eggs removed from egg papers prior to snap freezing samples for RNA extraction (see below).

Diapause incidence in both experiments was measured as described previously (Urbanski *et al.*, 2012). Briefly, at 11d post-oviposition, eggs on paper towels were stimulated to hatch by submersion in water. The number of larvae hatched was recorded for each replicate and the egg papers were re-dried. This process was repeated a second time. Remaining unhatched eggs were bleached to clear the chorion (Trpiš, 1970) and the number of fully developed, unhatched embryos was counted. Finally, diapause incidence was calculated as (no. embryonated unhatched eggs)/(no. hatched eggs + no. embryonated unhatched eggs).

Pharate larvae

Samples of pharate larvae tissue were generated by rearing F_{12} *Ae. albopictus* larvae under LD conditions as described above. Upon pupation, four replicate adult cages were established under SD conditions to produce eggs with pharate larvae entering diapause and four replicate adult cages were established under LD conditions to produce eggs with non-diapause pharate larvae. Each cage consisted of approximately 90 females and 90 males.

Females were allowed to blood feed to repletion 14–21d post-eclosion as described above. Although female age varied by seven days within replicates prior to blood feeding, this variation is unlikely to impact the small RNA composition of samples as the developmental trajectory of oocytes is most strongly affected by time since blood feeding rather than female age (Clements, 1992). Five days post-blood meal, females were provided a brown oviposition cup half-filled with deionized water and lined with unbleached paper towel. Eggs were collected, dried, and stored as described above. All eggs, including those produced by females reared under LD photoperiod, were stored under SD photoperiod. Exposure of eggs to an SD photoperiod does not induce diapause in *Ae. albopictus* (Mori &

Oda, 1981) and this approach was taken to minimize potential environmental effects of storage in different incubators. Eleven days post-oviposition, eggs were gently brushed from the unbleached paper towels into a 1.5mL tube, snap frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction and sequencing

RNA was extracted from both mature oocytes and pharate larvae by homogenizing tissue in TRI Reagent (Sigma-Aldrich, St. Louis, Missouri, USA) and performing a phenol-chloroform extraction followed by an isopropanol precipitation according to the manufacturer's directions. Residual DNA was removed from each sample using TurboDNA Free (Ambion, Austin, Texas). RNA integrity and concentration were assessed on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Individually-barcoded sequencing libraries were prepared according to the TruSeq RNA sample preparation kit (Illumina, San Diego, California, USA) at the University of Maryland Institute for Genome Science. All six barcoded mature oocyte samples (3 diapause, 3 non-diapause) were pooled and run on a single Illumina MiSeq lane. All eight barcoded pharate larvae samples (4 diapause, 4 non-diapause) were pooled and then split evenly on two Illumina MiSeq lanes.

Read Cleaning

Raw reads were processed in several steps prior to analysis. First, raw reads were screened using SSAHA2 (Ning et al. 2004) to remove tRNA (NCBI accession AY072044.1), rRNA (NCBI accession L22060.1), adapter and primer sequences. Remaining reads were trimmed to their longest continuous sequence with a quality score >30 at each base and reads <18nt were removed using the DynamicTrim and LengthSort scripts, respectively (Cox *et al.*, 2010)

miRNA annotation and differential abundance

In order to identify putatively novel miRNAs, reads obtained across all experiments and treatments were pooled and aligned to the *Ae. albopictus* genome (Chen *et al.*, 2015) using miRDeep2 (Friedländer *et al.*, 2008, 2012). Then, the 110nt region around each alignment was excised and scored for miRNA-like properties based on two criteria: [1] if the excised region can be folded into a stable hairpin precursor structure and [2] if reads aligned to the excised region match expected pre-miRNA splice sites. Next, alignments and structures were randomly permuted to estimate a true-positive rate across a range of miRDeep2 scores. Predicted miRNAs were considered putative novel miRNA precursors if they met the following four criteria: [1] achieved a miRDeep2 score >4 (corresponding to a true positive rate of 76±8%); [2] the length of the precursor miRNA hairpin loop was within the range of hairpin loop lengths observed in known *Ae. albopictus* miRNAs (16–145bp); [3] produced at least 10 mature reads from each arm across our 14 combined libraries; and [4] appeared in at least two separate libraries. The 20 mature miRNAs (derived from 10 precursor miRNAs) meeting these criteria were added to 237 previously annotated *Ae. albopictus* miRNAs (Skalsky *et al.*, 2010; Gu *et al.*, 2013) to produce a final reference set (Table S4).

Next, replicate libraries from each experiment and treatment were individually mapped to genome by using miRDeep2 and read counts for each mature miRNA in the final reference

set were obtained. For each experiment, read counts from all replicate libraries were TMM-normalized using the *edgeR* package (Robinson *et al.*, 2010) and only mature miRNAs present in at least half the libraries were retained. A distance matrix of miRNA expression patterns was visualized in a multi-dimensional scaling (MDS) plot using *PlotMDS* in the *limma* package (Ritchie *et al.*, 2015). Variation within experimental treatment groups was quantified with the biological coefficient of variation (BCV) using the *edgeR* package. Differential abundance of miRNAs in diapause compared to non-diapause was determined at both the oocyte and pharate larval stage using the *ExactTest* function in the *edgeR* package. MicroRNAs were considered differentially abundant if they had a Benjamani-Hochburg corrected p-value <0.05.

qRT-PCR validation

qRT-PCR was used to validate our RNA-Seq results at the pharate larval stage. The six miRNAs selected for validation include two miRNAs with significantly greater abundance during diapause (*aal-miR-283-5p* and *aal-bantam-5p*), two miRNAs with significantly lower abundance during diapause (*aal-miR-2942-3p* and *aal-miR-1-3p*), and two miRNAs with no significant difference in abundance during diapause (*aal-miR-133-3p* and *aal-miR-315-5p*). Therefore, the results of the qRT-PCR test the correspondence of qRT-PCR and RNA-Seq across the range of fold change values obtained during our RNA-Seq experiment.

At least 460 eggs were collected from six biological replicates (three SD conditions; three LD conditions) and snap frozen 11dpov as described above. Total RNA was extracted from each biological replicate as described above for the RNA-Seq experiments. Mature miRNA was reverse transcribed with the miScript II RT Kit with HiSpec buffer (Qiagen, Germantown, Maryland, USA) per the manufacturer's directions.

Relative abundance for each target miRNA was measured using the miScript SYBR Green PCR Kit (Qiagen, Germantown, Maryland, USA) on a Bio-Rad CFX96 Touch (Bio-Rad, Hercules, California, USA). Cycling parameters were 94°C for 15 min followed by 40 cycles of 94°C for 15s, 55°C for 30s, and 70°C for 30s. The miScript system utilizes a miRNA-specific forward primer and a proprietary universal reverse primer which binds to a sequence affixed to the -3p end of the mature miRNA during the reverse transcription process. For each target miRNA, we ordered one miRNA-specific miScript Primer Assay (Qiagen, Germantown, Maryland, USA). These primers were designed to align to the most abundant isomer of a particular miRNA in the RNA-Seq data (primer sequences are provided in Table S5).

For each target miRNA, the number of threshold cycles (C_t) was determined by averaging across three technical replicates per biological replicate. Relative abundance was calculated using the $2^{-\Delta\Delta Ct}$ method with the geometric mean of C_t values for *aal-miR-10-5p* and *aal-miR-87-3p* as a reference. Finally, we calculated the Pearson correlation coefficient for fold change values determined in this qRT-PCR experiment versus fold change values obtained during the RNA-Seq experiment.

Target prediction of differentially abundant miRNAs

The potential regulatory functions of differentially abundant miRNAs were investigated by identifying miRNA target sites using three algorithms with different methods of target prediction: miRanda (Enright *et al.*, 2003), RNAHybrid (Krüger & Rehmsmeier, 2006), and TargetScan (Lewis *et al.*, 2005). Only targets predicted by at least two of the three algorithms were retained as putative targets. Although somewhat conservative, this strategy is likely to reduce false positives and produce a high confidence set of targets (Witkos *et al.*, 2011). miRNA target sites are disproportionately located in the 3′ UTR region of mRNAs (Hausser & Zavolan, 2014), therefore 6,438 genes with annotated 3′ UTR regions in the *Ae. albopictus* genome were scanned for miRNA target sites.

miRanda

MiRanda uses a two-phase scoring algorithm to identify miRNA targets. First, potential targets for differentially abundant miRNAs are identified by aligning complementary miRNAs and 3′ UTRs. Alignments are scored by a position-weighted algorithm which favors matches in the seed region (miRNA nucleotide positions 2–8). Second, the free energy of each alignment is calculated to predict if interactions are thermodynamically favorable. MiRanda predictions were generated with the following settings: score cutoff 140, energy cutoff −20, gap open penalty −9, gap extension penalty −4.

RNAHybrid

RNAHybrid predicts targets by predicting the free energy of interaction between miRNAs and 3′UTR sequences. For each differentially abundant miRNA, the *RNACalibrate* module was run to estimate a null distribution for expected free energy of interaction for that miRNA against a random set of target sequences. Calibration output was subsequently used to run the main *RNAHybrid* module with the following settings: binding required in miRNA positions 2–7, p-value < 0.1, maximum target sequence length 100000, energy cutoff −20.

TargetScan

TargetScan identifies targets first by sequence alignment between differentially abundant miRNAs and 3′UTRs. Alignments are then checked for target conservation in a set of related species; in our analysis, conservation was identified by comparison to annotated 3′ UTR regions in three mosquito genomes: *Ae. aegypti*, *Culex pipiens*, and *Anopheles gambiae* (AegL3.3, CpipJ2.2, AgamP4.3, respectively, from VectorBase) as well as *D. melanogaster* (FB2016_03 from FlyBase). These four 3′UTR sets were aligned in a pairwise fashion to the *Ae. albopictus* 3′UTRs. For each species, the best hit for each 3′UTR was retained to generate a multiple sequence alignment file of 3′UTR putative orthologs. Next, putative orthologs for each differentially abundant miRNA were identified for each of the four Dipteran species using the sequence search tool on miRBase (Kozomara & Griffiths-Jones, 2014). Finally, this information on putative orthologous miRNAs and targets was provided to TargetScan and the algorithm was run with the default settings to identify conserved miRNA-target sites. Targets with 7nt or 8nt seed matches and conservation of nucleotides in the target seed region in at least one other species were retained.

Gene Ontology (GO) classification and visualization

To characterize the biological processes potentially affected by differentially abundant miRNAs, gene models predicted as miRNA targets by at least two algorithms were classified according to their associated Gene Ontology (GO) terms. GO terms were downloaded from VectorBase for each putative target. This list of GO terms was then consolidated to a simplified set of GO-Slim2 terms via the online CateGOrizer tool (Hu *et al.*, 2008) to provide a concise overview of biological processes for target genes. During this process, each GO-Slim2 term retains a count of how often sub-terms appear in the initial set.

GO-Slim2 terms and associated frequency counts were visualized in REVIGO (Supek *et al.*, 2011), which organizes GO terms by semantic similarity. The underlying semantic similarity algorithm, SimRel, uses the parent-child hierarchy of GO terms to assign similarity scores to pairs of GO terms based on the hierarchal distance between the pairs and how frequently each term appears overall in a given database. Pairs of GO terms which are closer together in the GO term hierarchy and appear less frequently receive higher similarity scores. Groups are formed when sets of GO terms receive similarity scores above a user-defined cutoff. Similarity scores >0.5 are considered functionally related (Schlicker *et al.*, 2006) and 99% of randomly generated GO term pairs score <0.53 (Supek *et al.*, 2011). Accordingly, the cutoff for similarity grouping was set to 0.5. *D. melanogaster* was chosen as a comparable GO term database for algorithm calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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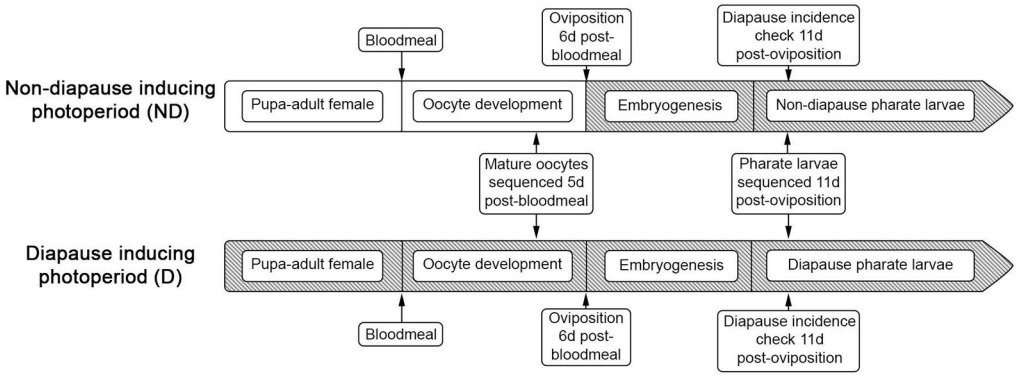

Figure 1.

Overview of experimental design. Unshaded portions of the timelines indicate exposure to long-day photoperiod (16h light:8h dark). Shaded portions of the timelines indicate exposure to short-day photoperiod (8h light:16h dark).

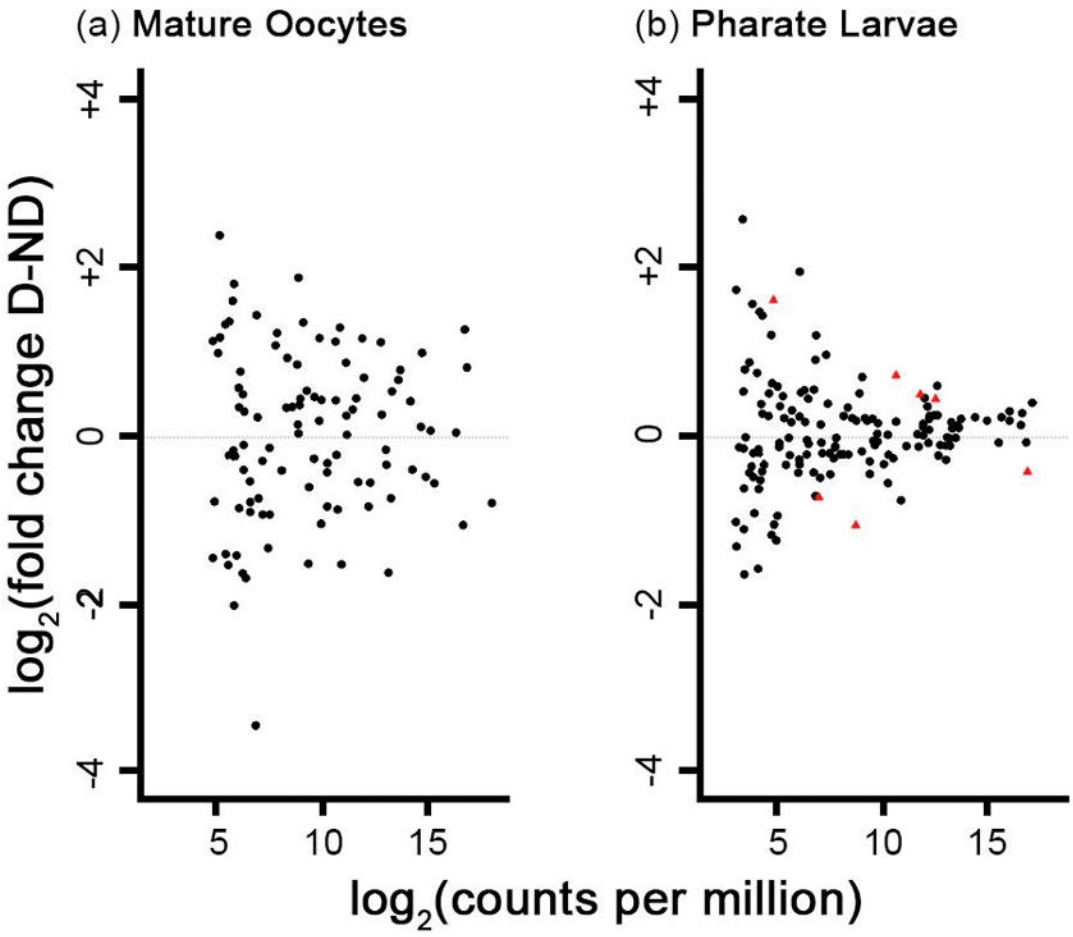

Figure 2.

Log_2(fold change) of miRNA abundance under diapause relative to non-diapause conditions versus abundance log_2(counts per million) following TMM-normalization for (a) mature oocytes and (b) pharate larvae. Each point represents a single miRNA. MicroRNAs with higher abundance under diapause conditions have positive fold change values. Red triangles indicate significant differences in miRNA abundance between conditions (Fisher's exact test $p<0.05$ following Benjamini-Hochberg correction).

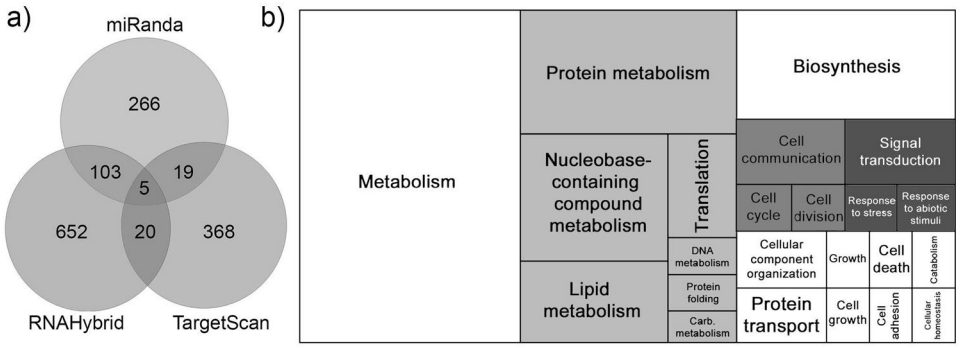

Figure 3.

(a) Count of 3′ UTR miRNA targets predicted by three algorithms for differentially abundant miRNAs. Targets predicted by at least two algorithms were retained for further investigation. (b) GO-Slim2 terms for predicted targets of differentially abundant miRNAs. The size of the square represents how frequently the term appears in annotations of genes with 3′UTRs targeted by differentially abundant miRNA. Groups of shaded squares are semantically similar subsets of terms as determined by REVIGO (SimRel score > 0.5). Predicted miRNA targets, target GO-Slim2 terms, and algorithms identifying particular miRNA-target pairs are detailed in Table S7.

Table 1

Summary of miRNA sequencing, cleaning, and mapping.

Sample	Number of Reads				
	Sequenced	**Cleaned Reads**	**Mapped to Genome**	**Mapped to Reference miRNAs**	**Mapped to Putative Novel miRNAs**
All Samples	57,432,813	12,895,405	7,303,656	3,348,144	57,254
Mature Oocytes					
Diapause 1	1,964,986	530,966	265,757	78,529	2,401
Diapause 2	2,470,716	1,008,763	472,872	88,760	14,497
Diapause 3	2,943,828	704,207	355,739	113,985	4,624
Non-diapause 1	2,360,151	818,099	379,917	48,049	7,710
Non-diapause 2	2,738,026	1,815,875	868,614	84,265	14,907
Non-diapause 3	2,387,982	1,529,625	752,327	99,268	13,259
Pharate Larvae					
Diapause 1	4,374,433	1,131,877	786,366	554,405	1,303
Diapause 2	5,241,029	895,362	579,820	391,158	1,066
Diapause 3	5,023,940	948,284	657,767	490,837	1,333
Diapause 4	6,195,237	804,382	546,724	387,633	1,204
Non-diapause 1	5,413,799	599,212	379,394	249,619	569
Non-diapause 2	4,870,514	789,399	484,299	283,740	585
Non-diapause 3	4,949,601	650,623	395,246	248,802	614
Non-diapause 4	6,498,571	668,731	378,814	229,094	568

Table 2

MicroRNAs with significant differences in abundance in diapause relative to non-diapause pharate larvae.

miRNA	log_2-FC	p-value	FDR
aal-miR-283-5p	+0.41	6.78e-04	0.035
aal-bantam-5p	+0.68	7.20e-04	0.035
aal-miR-2942-3p	−1.08	9.33e-04	0.035
aal-miR-286b-3p	+0.46	1.09e-03	0.035
aal-miR-282-5p	+1.58	1.12e-03	0.035
aal-miR-14-5p	−0.75	1.87e-03	0.046
aal-miR-1-3p	−0.45	2.07e-03	0.046