Changes in microRNA abundance may regulate diapause in the flesh fly, *Sarcophaga bullata*

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ABSTRACT

Diapause, an alternative developmental pathway characterized by changes in developmental timing and metabolism, is coordinated by molecular mechanisms that are not completely understood. MicroRNA (miRNA) mediated gene silencing is emerging as a key component of animal development and may have a significant role in initiating, maintaining, and terminating insect diapause. In the present study, we test this possibility by using high-throughput sequencing and qRT-PCR to discover diapause-related shifts in miRNA abundance in the flesh fly, *Sarcophaga bullata*. We identified ten evolutionarily conserved miRNAs that were differentially expressed in diapausing pupae compared to their nondiapausing counterparts. miR-289-5p and miR-1-3p were overexpressed in diapausing pupae and may be responsible for silencing expression of candidate genes during diapause. miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-275-3p, miR-276a-3p, miR-277-3p, and miR-305-5p were underexpressed in diapausing pupae and may contribute to increased expression of heat shock proteins and other factors necessary for the enhanced environmental stress-response that is a feature of diapause. In *S. bullata*, a maternal effect blocks the programming of diapause in progeny of females that have experienced pupal diapause, and in this study we report that several miRNAs, including miR-263a-5p, miR-100-5p, miR-125-5p, and let-7-5p were significantly overexpressed in such nondiapausing flies and may prevent entry into diapause. Together these miRNAs appear to be integral to the molecular processes that mediate entry into diapause.

Key Words: epigenetics, maternal effect, insects, seasonality

1. INTRODUCTION

Understanding the physiological, biochemical, and molecular basis of diapause is critical for predicting changes in the geographic and seasonal distributions of insects. Diapause is a phenotypically plastic, alternative developmental pathway that allows insects, and some other animals, to escape predictable periods of harsh environmental conditions and provides a way to synchronize periods of growth and reproduction with seasons of optimal conditions. The diapause program is initiated by token cues (e.g. changes in photoperiod, food quality, or temperature) that signal the coming of adverse conditions and leads to developmental arrest, metabolic depression, and increased-stress resistance. Physiological aspects of diapause (e.g. accumulated energy stores and changes in oxygen consumption rates) have been characterized for a variety of insects including crickets (Reynolds and Hand, 2009a), mosquitoes (Reynolds *et al.*, 2012; Zhou and Miesfeld, 2009), and butterflies (Lehmann *et al.*, 2016); and a growing body of literature documents changes in gene expression that drive the diapause program in arthropod species such as spider mites (Byron *et al.*, 2013), crickets (Reynolds and Hand, 2009b), locusts (Tu *et al.*, 2015), moths (Wadsworth and Dopman, 2015), bees (Amsalem *et al.*, 2015; Yocum *et al.*, 2015), mosquitoes (e.g. Poelchau *et al.*, 2013, Tormey *et al.* 2015), and several higher dipteran species (Flannagan *et al.*, 1998; Kubrak *et al.*, 2014; Ragland *et al.*, 2010, 2011). However, many details of the regulatory networks that initiate, maintain, and terminate diapause remain undefined. One particular gap in our understanding of diapause is how regulatory noncoding RNAs (e.g. long noncoding RNAs, small interfering RNAs, piwi-interacting RNAs, or miRNAs) regulate diapause. The focus of our present research is to discover potential contributions of miRNAs (miRNAs) in regulating pupal diapause in the flesh fly, *Sarcophaga bullata*.

MiRNAs are 18-25 nucleotide (nt) RNAs that post-transcriptionally regulate gene expression through their interactions with target gene transcripts. They have been implicated as regulators of numerous diapause-relevant biological processes including developmental timing (Hammel *et al.*, 2009;

Abbot *et al.*, 2005), cell-cycle progression (Brennecke *et al.*, 2003, Zhao *et al.*, 2015), metabolism (Teleman, *et al.*, 2006; Ramirez *et al.*, 2013), and stress-resistance (Barrio *et al.*, 2014; De Lella Ezcurra, *et al.*, 2016). Changes in miRNA abundance are known to be associated with other types of dormancy including nematode dauer formation (Karp and Ambrose, 2012; Karp *et al.*, 2011; Than *et al.*, 2013) and mammalian hibernation (Hadj-Moussa *et al.*, 2016; Kornfield *et al.*, 2012; Lang-Oulette and Morin, 2014; Wu *et al.*, 2014, 2016). Changes in miRNA profiles have been observed in response to environmental stressors including extreme cold, dehydration, and anoxia (Biggar *et al.*, 2012; Chen *et al.*, 2013; Courteau *et al.*, 2012; Lyons *et al.*, 2015). Taken together, these reports on other animals suggest the possibility that miRNA may have a significant role in establishing and maintaining multiple aspects of insect diapause.

Preliminary evidence for miRNA involvement in the pupal diapause of *S. bullata* comes from our previous experiments showing diapause-related changes in the abundance of genes encoding core components of the miRNA silencing pathway (Reynolds *et al.*, 2013). Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II as single-strands that can fold into a characteristic hairpin structure. Pri-miRNAs are cleaved by Drosha and become miRNA precursors (pre-miRNAs) that are exported to the cytoplasm where they are further processed by Dicer, resulting in two mature miRNAs from the 5' and 3' arms of the hairpin. Mature miRNAs bind to the RNA-induced silencing complex (RISC) and guide it to a target mRNA that contains a complementary sequence that is often found in the 3' UTR but may also be located in the 5' UTR or gene body (Schnall-Levin *et al.*, 2010). The RISC silences the target gene by either inhibiting translation or promoting transcript degradation. In diapausing *S. bullata* the gene encoding Argonaute1 (Ago1), the catalytic core of the RISC, is upregulated more than 2-fold compared to levels observed in nondiapausing pupae. *Ago1* transcript abundance is downregulated more than 50% within 24 h after treating pupae with hexane to terminate diapause. These significant changes in *Ago1*

transcript abundance suggest a role for miRNA-mediated gene silencing that is lifted once diapause is terminated (Ha and Kim, 2014; Reynolds et al. 2013, Tomari et al., 2007).

The current study builds on our previous work by using high-throughput sequencing and quantitative reverse transcription PCR (qRT-PCR) to assess diapause-related shifts in miRNA abundance, thereby identifying specific miRNAs that may regulate *S. bullata* diapause. Illumina sequencing of small-RNA libraries constructed with total RNA from diapausing and nondiapausing *S. bullata* allowed us to identify conserved miRNAs that are present during this stage of development and to profile miRNA abundances in dormant and direct-developing flies. Measurements of candidate miRNAs with qRT-PCR confirmed Illumina sequencing results and allowed us to assess miRNA abundance in additional developmental stages including post-diapause pharate adults. We also used qRT-PCR to measure miRNA abundance in pupae that were not in diapause because of a maternal effect that blocks diapause entry (Henrich and Denlinger, 1982; Rockey *et al.*, 1989). Our results show changes in abundance for a number of evolutionarily conserved miRNAs that we think are likely to have a significant role in regulation of diapause in *S. bullata* and expression of the maternal effect.

2. METHODS

2.1 Insect Rearing and sampling

S. bullata in our established laboratory colony were reared as previously described (Denlinger 1972; Reynolds *et al.* 2013). Adults were provided unlimited access to water and sucrose; beef liver was provided as a protein source for the first 7 d after adult emergence. Adults were maintained under either short-day conditions (25 °C; 8 h photophase) that induce diapause in their progeny or long-day conditions (25 °C, 16 h photophase) that prevent diapause induction. Adults were provided fresh beef liver as a larviposition site 11-13 d after adult emergence. Newly deposited larvae were transferred to aluminum foil packets containing sufficient liver to allow completion of larval development; aspen

SaniChips (Harlan, Indianapolis, IN, USA) were provided as a pupariation site. All larvae were reared at 18 °C; pre-diapause larvae were kept under a short-day photoperiod while larvae not programmed for diapause were maintained under a long-day photoperiod. Both diapausing and nondiapausing pupae were sampled once they reached the phanerocephalic stage (i.e., the stage when diapause is entered) which occurs approximately 10 d post-larviposition at 18 °C.

Post-diapause flies were generated by removing the anterior cap of the puparium and applying 5 µl hexane to the exposed head to terminate diapause (Denlinger *et al.*, 1980). Hexane-treated flies were kept at 25 °C with a long-day photoperiod until adult emergence, with sampling 24 and 48 h post-treatment as well as on the day pharate adult flies reached the red-eye stage, approximately 2 weeks post-treatment.

To elicit the maternal effect, adults that had experienced diapause as pupae were treated with hexane to terminate diapause and upon emergence were maintained under short-day conditions; larvae collected from such females were also held under short days at 18 °C and pupae were sampled when they reached the phanerocephalic stage.

2.2 Illumina sequencing and miRNA identification

Small RNA libraries were constructed from total RNA isolated from diapausing or nondiapausing pupae using the *mirVana*[™] miRNA Isolation Kit (ThermoFisher, Waltham MA, USA) according to the manufacturer's instructions for RNA isolation from fresh tissue. RNA was isolated from multiple individuals and pooled. Prior to sample pooling, the concentration of each RNA sample was assessed using a Nano Drop spectrophotometer (ThermoFisher, Waltham MA, U.S.A.), and equivalent amounts of RNA from 3 separate individuals were combined prior to construction of each library. Three libraries were constructed and sequenced for each pupa type (diapause or nondiapause).

Library construction and Illumina sequencing were carried out by LC Sciences (Houston, TX, USA). After a quality check for each sample, libraries were prepared using the Illumina Truseq[™] Small

RNA Preparation kit according to the sample preparation guide. Purified cDNA libraries were sequenced on Illumina GAllx according to the vendor's standard operating procedures. Raw sequence reads of 40 nucleotides were obtained using Illumina's Sequencing Control Studio Software version 2.8 following real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70.

Extracted sequence reads were used in standard data analysis using a proprietary pipeline script, ACGT101-miR v4.2 (LC Sciences). Raw sequence reads (i.e. sequenced sequences) were clustered into unique families, then low quality sequences were removed. The remaining sequences were grouped into sequence families (i.e. unique sequences) and were mapped to reference databases including RepBase (Jurka *et al.*, 2005), and RFam (Gardner *et al.* 2009) to identify and remove sequences that mapped to other types of small RNAs. Remaining sequences were mapped to precursor miRNA (pre-miRNAs) and mature miRNA sequences from Arthropods in miRBase release 21 (Kozomara and Griffiths-Jones, 2013) and to an unpublished *S. bullata* draft genome (J. Peyton and D. Denlinger, unpublished data).

2.3 Quantitative Reverse-transcription PCR

qRT-PCR was used to measure candidate miRNAs in additional developmental stages. Total RNA was isolated using the *mirVana*[™] miRNA Isolation kit as described above. Reverse transcription of miRNAs was carried out using the miScript II RT Kit (Qiagen, Valencia, CA, USA) according to manufacturer's directions for using the HiSpec buffer, which specifically transcribes mature miRNAs. Two micrograms of total RNA were used for each reaction.

Relative abundance of each miRNA of interest was measured using an iQ5[™] Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA), along with the miScript SYBR Green PCR Kit and miScript Primer Assays (Qiagen), which used a combination of one universal primer and one primer that was custom designed to detect a specific *S. bullata* miRNA sequence. Primer performance conformed to MIQE Standards (Bustin *et al.*, 2009) as shown in Supplementary Table S1). Cycling

parameters were 94 °C for 15 m followed by 40 cycles of 94 ° for 15 s, 55 °C for 30 s and 72 °C for 30 s. Melt curve analysis indicated only one product was formed under these conditions.

Relative miRNA abundance was evaluated in 6-7 replicate samples for each group with three technical replicates for each miRNA assay. A modified $2^{-\Delta Ct}$ method was used to calculate replicates for miRNA of interest (Reynolds *et al.*, 2013). Briefly, we averaged the threshold cycles (C_t) of the technical replicates for each replicate sample, then subtracted the geometric means of the C_t for the appropriate reference primers which depended on the abundance of the candidate miRNA. The geometric mean of U6 snoRNA and miR-8-3p was used to normalize the results of high abundance miRNAs; miR-8-3p and miR-10-5p were used to normalize low abundance miRNAs. It should be noted that there were no significant differences in the qRT-PCR measurements for miR-8-3p even though the sequencing results suggested this miRNA was differentially expressed in diapausing vs nondiapausing pupae (Supplementary Figure S1). The resulting value was log transformed to give relative miRNA abundance ($2^{-\Delta Ct}$). Student's t-test or Mood's Median test was performed using MiniTab software (MiniTab, State College, PA, USA) to compare the relative abundances as specified in the Results.

Data access

High-throughput sequencing data generated during this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nih.gov/geo/>) under accession number GSE94376.

3. RESULTS

3.1 Discovery of miRNAs in diapausing and nondiapausing pupae

We obtained 51,815 raw reads through Illumina sequencing of small RNA libraries that were constructed from total RNA isolated from ontogenetically matched diapausing and nondiapausing pupae.

Approximately 92 % of the reads could be mapped to publicly available databases. Filtering out low quality sequences, reads that lacked the 3' adaptor sequences, and reads that were less than 15 nt gave 47,814 mappable reads that were 15-45 nt in length. Approximately 12 % of these were 18-25 nt in length (Fig. 1), the size typically associated with miRNAs. This proportion of miRNA-sized sequences is lower than expected compared to results of small RNA-Seq analyses for some other insects (e.g. Khajuria *et al.*, 2013; Tuckow *et al.*, 2013; Wei *et al.*, 2009) but is consistent with those measured for *Bombyx mori* in the pupal stage (Jagadeeswaran *et al.*, 2010) and may be typical for this stage of development. Surprisingly, the majority of small RNAs sequenced from *S. bullata* pupae were 28-30 nt long, which is the size typical for piwi-associated RNAs (piRNAs), another class of small regulatory RNAs (Seto *et al.*, 2007). A similar proportion of reads in this size range was previously reported for *Bombyx mori* pupae (Jagadeeswaran *et al.*, 2010), thus it is likely that these small RNAs are necessary and important for holometabolous metamorphosis. However, the role of piRNAs as regulators of pupal development is beyond the scope of this study.

Mappable reads in the 15-32 nt range were clustered into families of unique sequences that were mapped to public sequence databases including miRBase (Kozomara and Griffiths-Jones, 2013), RepBase (Jurka *et al.*, 2005), RFam (Gardner *et al.* 2009) and to an unannotated draft of the *S. bullata* genome (J. Peyton and D. Denlinger, unpublished data). Approximately 1,400 unique sequence families, or types, were defined from 248,153 sequences sequenced (*i.e.* individual reads; Table 1). Of the mappable reads, approximately 3 % (172,895 sequences) mapped to mature and/or precursor Arthropod miRNAs found in miRBase version 21 (*i.e.* Groups 1, 2, and 3); an additional 0.2 % (75,258 sequences) were predicted novel miRNAs that may be unique to *S. bullata* (Group 4). Clustering these sequences allowed us to identify 1,297 evolutionarily conserved miRNAs and 104 predicted miRNAs, which are presented in Supplementary Tables S2-S6). Remaining sequences mapped to RFam or

RepBase (32,828,539 sequences) or to regions of the *S. bullata* genome that could not form the hairpin structure indicative of miRNA precursors (10,333,809 sequences).

The twenty-four most abundant sequence families represent both evolutionarily conserved miRNAs and predicted novel miRNA sequences (Fig. 2). All of the most highly abundant sequences were present in both diapausing and nondiapausing pupae, but the rank order of most sequences differed between the two groups. The five most abundant sequences in nondiapausing pupae were, in order, miR-276a, PC-3p-4142, miR-282-5p, PC-5p-1003, and miR-8-3p. In diapausing pupae, PC-3p-4142, PC-5p-1003, miR-267a, PC-5p-5395, and mir-6497-5p_1ss5AG were the most abundant. Eleven highly abundant sequences were present in significantly different amounts in nondiapausing vs. diapausing pupae. Sequences more abundant in nondiapausing pupae included miR-8-3p ($P = 0.037$), miR-31a-5p ($P = 0.002$), miR-305-5p ($P = 0.017$), and miR-276a ($P = 0.002$). Mir-6479-5p_1ss15AG ($P = 0.003$), mir-6497-3p_1ss2GT ($P = 0.020$), mir-6497-3p_1ss13AG ($P = 0.002$), PC-5p-5395 ($P = 0.008$), PC-5p-1003 ($P = 0.020$), PC-3p-5997 ($P = 0.003$), and PC-3p-4142 ($P = 0.002$) were more abundant in diapausing pupae.

The majority of highly abundant sequences were considered to be evolutionarily conserved because they mapped to miRNAs known from other Arthropods. Five highly abundant sequences did not map to sequences in miRBase, thus they were classified as putative novel miRNAs that could be unique to *S. bullata*. Criteria for classification as *bona fide* miRNAs include being the correct size (18-25 nt), mapping to a region of the genome that has the potential to fold into a hairpin structure typical of a miRNA precursor, and having a minimum free energy below a specific threshold (Ambrose *et al.* 2003). Additional work is needed to confirm that these sequences are indeed miRNAs and to elucidate their possible functions. Thus, we chose not to include further discussion of these putative novel miRNAs in this report on miRNA regulation of diapause. We also identified several isomiRs of miR-2779 and mir-6497 in *S. bullata* pupae. IsomiRs contain slight sequence variations relative to a reference sequence (Morin *et al.*, 2008), usually a single nucleotide substitutions or the addition or subtraction of a few

nucleotides to the 3' or 5' end of the miRNA sequence. Although mir-6497 and miR-2779 isomiRs were highly abundant in diapausing pupae compared to nondiapausing pupae, their function is unknown and their significance is beyond the scope of this study. The remainder of this paper will focus on differences in the abundance of evolutionarily conserved miRNAs in diapausing and nondiapausing *S. bullata* and their possible regulatory functions.

3.2 Differences in miRNA abundance related to diapause and the maternal block of diapause

qRT-PCR was used to measure abundance of candidate miRNAs identified with RNA-Seq in additional diapause states and developmental stages. Specifically, we used qRT-PCR to measure differences in miRNA abundance that may be associated with expression of the maternal effect that blocks diapause entry in this species. We also measured shifts in miRNA abundance following diapause termination. Candidate miRNAs (miR-31a-5p, miR-305-5p, miR-276a-3p, miR-1-3p, miR-13b-3p, miR-190-5p, miR-305-5p, miR-276b-3p, miR-100-5p, miR-9c-5p, miR-289-5p, miR-92b-3p, miR-125-5p, miR-275-3p, miR-263a-5p, and let-7) were selected because sequencing results indicated they were differentially expressed in diapausing pupae compared to their nondiapausing counterparts (Fig. 3) and/or because reports from other insects, namely *Drosophila melanogaster*, indicate they have possible diapause-related functions (e.g. miR-277-3p).

S. bullata reared under diapause-inducing short-day photoperiods as embryos and first-instar larvae enter diapause as pupae. An exception occurs when female *S. bullata* that have a diapause history (i.e. experienced diapause themselves, DH) produce offspring that are not able to enter diapause even when reared under strong diapause-inducing conditions (18 °C, short-day photoperiod). We measured abundance of 15 candidate miRNAs in diapausing pupae (NDH-SD, no maternal diapause history, short-day rearing environment) compared to ontogenetically matched nondiapausing pupae that either did not enter diapause because they were reared in a diapause-averting environment (NDH-LD, no maternal diapause history, long-day rearing environment) or could not enter diapause because of

their mother's diapause history (DH-SD, diapause maternal history, short-day rearing environment). The goal of this set of experiments was to identify miRNAs that may regulate the diapause and the maternal block of diapause by asking two questions: 1) does miRNA abundance differ between diapause and the two nondiapause groups; 2) does miRNA abundance differ between the two nondiapause groups? Results from qRT-PCR analyses allowed us to assign miRNAs to one of 5 categories – 1) miRNAs that were over- or underexpressed in diapausing pupae compared to both nondiapausing groups; 2) miRNAs with equivalent, or nearly equivalent amounts in flies from mothers with no diapause history (NDH-SD and NDH-LD) but were over- or underexpressed in DH-SD pupae that expressed the maternal effect; 3) miRNAs with equivalent amounts in short-day reared flies (NDH-SD and DH-SD) but a different amount in NDH-LD flies; 4) miRNAs that were present in significantly different amounts in all three groups, 5) miRNA abundance was equivalent (*i.e.* no significant differences) in all three groups. Category 1 miRNAs (Fig. 4) included miR-1-3p, miR-13b-3p, miR-31a-5p, miR-275-3p, miR-276a-3p, and miR-305-5p. MiR-9c-5p, let-7, miR-100-5p, miR-125-5p, and miR-263a-5p were in category 2 (Fig 5). Category 3 included miR-277-3p and miR-289-5p (Fig. 6). Categories 4 and 5 included miR-92b-3p (Fig. 7a) and miR-190-5p (Fig. 7b), respectively.

3.3 Changes in miRNA abundance at diapause termination and post-diapause development

Diapause in *Sarcophaga spp.* can be terminated pharmacologically by applying hexane to the heads of diapausing pupae (Denlinger *et al.*, 1980). We used qRT-PCR to assess changes in the abundance of five candidate miRNAs 24 and 48 h after hexane application. Only miR-31a-5p, and miR-289-5p were significantly reduced 24 h following diapause termination; the median value of let-7 abundance was unchanged 24 h post-diapause then appeared to increase 48 h post-diapause (Fig. 8). MiR-305-5p and miR-13b-3p remained unchanged even 48 h post-diapause ($P = 0.513$ for both miRNAs).

In *C. elegans* dormancy-related (*i.e.* dauer-related) changes in miRNA abundance can persist into adulthood and have long-term effects on life history (Karp *et al.*, 2011). This post-dauer effect prompted

us to consider long-term changes in *S. bullata* that had experienced diapause. We compared abundance of candidate miRNAs in red-eye pharate adults that had previously been in diapause (i.e. had a diapause history) to red-eye pharate adults with no diapause history. We found significant increases in the abundance of let-7, miR-31a-5p, miR-125-5p, and miR-190-5p in red-eye pharate adults that had previously been in diapause compared to their nondiapause counterparts (Figure 9). Diapause history did not significantly influence the abundance of miR-9c-5p or miR-13b-3p.

3.4 Predicted targets of differentially expressed miRNAs

DIANA miRPath v.3.0 (Vlachos et al., 2015), TargetScanFly release 6.2 (Kheradpour, et al., 2007; Ruby et al, 2007), and miRanda (Betel et al., 2008), were used for *in silico* predictions of genes targeted by miRNAs that had altered expression profiles in diapausing pupae of *S. bullata*. DIANA miRPath was used to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa *et al.*, 2016) and Gene Ontology (GO) defined processes (Ashburner *et al.*, 2000). KEGG pathways with a high probability of being regulated by diapause-relevant miRNAs include Mucan type O-Glycan biosynthesis; Valine, leucine, and isoleucine degradation; MAPK signaling; Hippo Signaling; N-Glycan biosynthesis and other pathways shown in Table 2. GO defined biological processes likely regulated by differentially expressed miRNAs include Imaginal disc-derived wing morphogenesis, compound eye development, open tracheal system development, and others listed in Table 3.

miRPath analyses were also used to identify diapause-relevant miRNAs that may regulate KEGG pathways and Gene Ontology defined processes found to be enriched in microarray comparisons of “diapause and nondiapause” transcriptomes of *S. crassipalpis*, a species closely related to *S. bullata* (Ragland *et al.*, 2010). This analysis identified 32 putative gene targets for miR-1-3p, miR-13b-3p, miR-92b-5p, miR-263a-5p, miR-275-3p, miR-277-3p, and miR-289-5p in diapause-relevant pathways including DNA replication, mismatch repair, glycerolipid metabolism, ether lipid metabolism, pyruvate metabolism, citrate cycle, protein folding, defense response, carbohydrate metabolic process,

transaminase activity, response to stimulus, unfolded protein binding, and nuclear pre-replicative processes (Tables 4 and 5).

TargetScanFly and miRanda were used to identify miRNAs that may regulate individual genes, rather than pathways, that were over- or underexpressed in diapausing *S. crassipalpis* (Pavlidis *et al.*, 2011; Ragland *et al.*, 2010; Li *et al.*, 2007). Abundance of a specific miRNA is generally negatively correlated with abundance of its target genes, thus we focused on pairing overexpressed miRNAs with underexpressed mRNAs and vice versa (Table 6). TargetScanFly was used to identify miRNA binding sites in both the open reading frame (ORF) and 3' UTRs of candidate genes since miRNA target sites are found within both ORFs and 3' UTRs in *D. melanogaster* (Schnall-Levin *et al.*, 2010). Some notable, diapause-relevant miRNA:mRNA interactions predicted by TargetScaFly and miRanda include the miR-9c-5p regulation of the genes encoding *phosphoenolpyruvate carboxykinase (pepck)* and heat shock protein *dnaj*, miR-277-3p regulation of *heat shock protein (hsp) 70*, *hsp60*, and *hsp67*, and miR-305-5p regulation of *Acetyl coenzyme A synthase (AcCoAs)* and *Insulin-like receptor (InR)*.

miRPath analyses also identified pathways that may be regulated by miRNAs that were differentially expressed in pupae that failed to enter diapause because of the maternal effect that blocks diapause entry compared to pupae that did not enter diapause because of their rearing environment. Pathways targeted by let-7-5p, miR-100-5p, miR-125-5p, miR-263a-5p, miR-9c-5p, miR-289-5p, miR-277-3p and miR-92b-3p included Mucin type O-Glycan biosynthesis, Valine, leucine and isoleucine degradation, MAPK signaling pathway, Biotin metabolism, Hippo signaling pathway, Phototransduction, and others listed in Table 7.

4. DISCUSSION

The profiles of miRNA abundances that we present here for diapausing and nondiapausing pupae of *S. bullata* identify evolutionarily conserved small RNAs that are candidate regulators of insect pupal

diapause. Using next-generation sequencing and qRT-PCR, we identified two evolutionarily conserved miRNAs (miR-1-3p and miR-289-5p) that were significantly overexpressed in diapausing pupae compared to their nondiapausing counterparts and eight miRNAs (miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-275-5p, miR-276-3p, miR-277-3p, and miR-305-5p) that were underexpressed in diapausing pupae. Finally, we identified seven miRNAs (miR-31a-5p, miR-190-5p, miR-9c-5p, let-7-5p, miR-100-5p, miR-125-5p, miR-263a-5p) that may regulate a maternal effect that blocks diapause entry in this species. Together, these results provide the first evidence for shifts in miRNA abundance associated with insect diapause.

We were surprised to find that the majority of differentially-expressed miRNAs were underexpressed during diapause and that only two miRNAs (miR-1-3p and miR-289-5p) were more abundant in diapausing pupae than in their nondiapausing counterparts. This result was unexpected because miRNA abundance has most often been negatively correlated with expression of its target genes. Thus, we anticipated that the majority of differentially expressed miRNAs would be overexpressed during diapause in conjunction with the large-scale reduction in gene expression that is a common feature of diapause in *S. bullata* and other insects (Denlinger, 2002). One possible explanation for this observation is that some miRNAs positively regulate their targets rather than inhibit their expression (Vasudevan et al., 2007), and reducing miRNA abundance could lead to a decrease in the transcription of the target genes. There are many examples of miRNAs stimulating gene expression in vertebrates (e.g. Mortensen et al., 2011; Place et al., 2008, Vasudevan et al., 2007). However, it is unclear how widespread this phenomenon is in insects because it has been reported only in locusts thus far (He et al., 2016). Additional studies will explore the possibility that miRNAs that were underexpressed in diapausing *S. bullata* are required for continuous development.

353 Though we did not observe a huge number of overexpressed miRNAs associated with pupal
354 diapause, we propose that the specific changes in abundance for each of the differentially expressed
355 miRNA that we observed reflect functions of target genes as discussed below.

356 Establishing diapause-related shifts in miRNA abundance is a critical first step in understanding
357 how these small RNAs may regulate diapause, but in order to understand the functional significance of
358 these observed changes, the biological processes of genes targeted by these differentially expressed
359 miRNAs must be identified. At this time, there are no confirmed targets for any *S. bullata* miRNAs, and
360 only a few miRNAs from any insect species have targets or functions that have been experimentally
361 verified. Thus, the functional relevance of differentially expressed miRNAs must be inferred from
362 published studies on other animals, (e.g. *Aedes aegypti* (Bryant et al., 2010); *Drosophila melanogaster*
363 (e.g. Chen and Robash, 2016 and studies below); *Caenorhabditis elegans* (Abbot et al., 2005), and
364 *Artemia parthenogenetica* (Zhao et al., 2015)) or from *in silico* predictions of miRNA targets made using
365 TargetScan Fly, miRanda, and DIANA-mirPath. Together these tools allowed us to develop hypotheses
366 about how differentially expressed miRNAs mediate developmental arrest, metabolic depression, and
367 stress-resistance associated with the diapause program in *S. bullata*.

368 Developmental arrest is a defining feature of diapause, and a number of miRNAs identified in
369 this study have been implicated in regulation of developmental processes including neurogenesis (the
370 miR-9 family, miR-289-5p, miR-277-3p, and miR-13b); stem cell proliferation and differentiation (miR-
371 305); developmental timing (let-7, miR-125) and cell cycle progression (miR-100). Of these, miR-289-5p,
372 miR-277-3p and miR-305 are notable because they regulate processes associated with metamorphosis
373 (see references below), the stage of development that is arrested when insects, including *S. bullata*,
374 enter diapause as pupae. MiR-289-5p regulates formation of neuromuscular junctions, which undergo
375 significant changes as larval tissues are degraded and new adult tissues are constructed. This miRNA,
376 which was overexpressed more than 2-fold during diapause, negatively regulates synapse growth by

377 targeting genes necessary for axon development and pathfinding (Liu *et al.*, 2010; Nesler *et al.*, 2013;
378 Sonea and Rhuben, 1992) and could potentially arrest formation of neuromuscular junctions during
379 diapause. MiR-305 regulates intestinal stem cell (ISC) proliferation and differentiation, an integral part of
380 gut remodeling during metamorphosis. In *D. melanogaster*, ISC proliferation and differentiation are
381 regulated by miR-305 through the targeting of genes in the insulin and Notch signaling pathways,
382 especially *insulin receptor (InR)* and *phosphatidylinositol-3-kinase (pi3K)* (Foronda *et al.*, 2014;
383 Parthasarathy and Palli, 2008), both of which are overexpressed during diapause in *S. crassipalpis*
384 compared to their nondiapausing counterparts (Ragland *et al.*, 2010). Additional miRNAs that may be
385 important regulators of developmental arrest in diapausing pupae include let-7, miR-100, and miR-125
386 which regulate developmental timing and cell-cycle progression in *Drosophila* and other insects (Caygill
387 and Johnston, 2008; Sempere *et al.*, 2003) and are important for metamorphosis in *Bombyx mori* (Ling *et*
388 *al.*, 2014). Although these miRNAs were not differentially expressed during diapause in *S. bullata* they
389 may still have a significant role in mediating these functions, a feature we hope to evaluate in the future.

390 MiRNAs are increasingly recognized for their role in regulating hypometabolic states in animals
391 ranging from sea cucumbers to bats (Biggar and Storey 2011; Chen *et al.*, 2013; Kornfeld *et al.*, 2012),
392 and they may contribute to the suppression of metabolism during diapause in *S. bullata*. Though we do
393 not have transcriptome data for diapausing *S. bullata*, a microarray study on *S. crassipalpis*, a closely
394 related species, provides some indication that select metabolic pathways may be regulated, at least in
395 part, by miRNAs in diapausing pupae of *S. bullata*. Using mirPath to integrate our miRNA data with
396 published *S. crassipalpis* transcriptome data suggests that glycerolipid metabolism, pyruvate
397 metabolism, citrate cycle, cellular carbohydrate metabolic processes, and riboflavin metabolism may be
398 regulated by miRNAs during diapause. These pathways were all enriched in transcriptomes of diapausing
399 *S. crassipalpis* (Ragland *et al.*, 2010) and contain genes that are predicted targets of miRNAs present in *S.*
400 *bullata*. Two miRNAs in particular, miR-277-3p and miR-289-5p, appear to have substantial influence

over diapause-related metabolic processes; each miRNA potentially regulates nine gene targets in at least 4 of the metabolic pathways listed above. Predicted gene targets of miR-289-5p encode components of all four metabolic pathways listed above and include CG6847, wun, CG34384, BBC, CG32699, CG1332, Men-b, CG10970, and ATPCL. ATP citrate lyase (*atpcl* or *acly*) is of particular interest because it has been implicated in glucose metabolism and life-span extension in *Drosophila* (Peleg *et al.*, 2016; Ugrankar *et al.*, 2015) and is downregulated by 75 % in diapausing *S. bullata* (Reynolds *et al.*, 2016). Future studies will aim to confirm interaction between these miRNAs and their predicted gene targets, as well as the significance of these miRNAs to metabolic regulation during diapause in *S. bullata*.

Changes in miRNA abundance that occur in the gall moth, *Epiblema scudderiana*, and the goldenrod gall fly, *Eurosta solidaginis*, in response to cold stress (Courteau, *et al.*, 2012; Lyons *et al.*, 2015) suggest a protective role for some miRNAs in diapausing *S. bullata*. Many of the cold-responsive miRNAs reported in these two studies, including miR-1-3p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-276a-3p, and miR-277-3p were also differentially expressed in diapausing *S. bullata*. Diapause and cold hardiness are linked in *S. bullata* (Adedokun and Denlinger, 1984), and we suspect that these miRNAs have a cold stress-response function that is evolutionarily conserved among insects, and possibly other ectotherms. Predicted targets of these miRNAs include *heat shock protein 60* (*hsp60*), *hsp-70Bbb*, and *hsp27* which are all upregulated in diapausing *S. crassipalpis* (Rinehart *et al.*, 2007; Ragland *et al.*, 2010) and are putative targets of miR-92b-3p, miR-276a-5p, miR-277-3p, respectively. MiR-277-3p may also regulate genes in multiple diapause-related stress response pathways identified in diapausing *S. crassipalpis* (Ragland *et al.*, 2010), including transaminase activity (GO:0008483), unfolded protein binding (GO: 51082), and chaperonin containing t-complex (GO:0005832). Future studies that manipulate abundance of these and other under-expressed miRNAs will allow us to assess their role in the diapause-associated stress response.

A maternal effect operating in *S. bullata* prevents diapause from occurring in the offspring of adult females that have experienced pupal diapause. Increased abundance of miR-31a-5p and miR-190-5p in red-eye pharate adults that experienced diapause compared to similar stage flies with no diapause history suggests these miRNAs may influence the maternal block of diapause in the next generation. Increased abundance of let-7-5p, miR-100-5p, miR-125, miR-263a-5p, and miR-9c-5p in DH-SD pupae that could not enter diapause due to this maternal effect, compared to nondiapause pupae that did not enter diapause because of their diapause-averting rearing environment, suggests that these miRNAs may contribute to regulation of the maternal effect. Predicted gene targets of these miRNAs are members of four KEGG defined pathways, including Glycosaminoglycan biosynthesis, Phototransduction, Spliceosome (i.e. RNA splicing), and Phagosome (i.e. genes involved in phagocytosis); but it is unclear what roles these pathways play in blocking diapause entry. Let-7, miR-100-5p, and miR-125-5p are regulated by ecdysone and prothoracicotropic hormone in *D. melanogaster* (Chen *et al.*, 2014; Semper *et al.*, 2002), and the upregulation of these miRNAs suggests increased titers of these hormones in pupae from diapause mothers. This is consistent with the low ecdysone titers that are essential for manifestation of pupal diapause in flesh flies (Denlinger, 1972; Richard *et al.*, 1987), as well as other insects that diapause as pupae (Denlinger *et al.*, 2002). Future examination of adult females that went through diapause during the developmental window for programming the maternal effect will be helpful for identifying possible miRNA links to ecdysone and other factors that could be regulating the maternal effect. The maternal block of diapause in short-day reared flesh flies offers an enticing challenge, and our results implicating miRNAs in diapause and the maternal effect suggest new paths of investigation for understanding these complex regulatory mechanisms.

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FIGURE LEGENDS

Figure 1. Size distribution and abundance of small RNAs in pupae of *S. bullata*. A) Abundance of each size of small RNA sequenced based on nucleotide (nt) length. B) Proportion of sequences meeting the size requirements for miRNAs.

Figure 2. Conserved and potentially novel miRNA sequences with the highest abundance in diapausing and developing pupae. All highly abundant sequences were found in samples from both diapausing and nondiapausing pupae, but some sequences were present in significantly different amounts as indicated by “*”. Sequences designated “miR-X” indicate they mapped to the mature sequence in miRBase, while sequences designated “mir-X” mapped to known miRNA precursors but were not perfectly mapped to the mature sequence. Sequences designated with “PC-###” are putative novel *S. bullata* miRNAs. Sequences with “1ss5AG” indicate an isomiR of a known miRNA sequence with the indicated nucleotide substitutions. Bars represent the mean number of reads normalized to the total number of reads. Significance was determined with Student’s t-test; bars marked with “*” were significantly different at $P \leq 0.05$. N = 3 replicate samples.

Figure 3. Sequence abundances of candidate miRNAs selected for measurements in additional developmental stages. The top graph depicts high abundance miRNAs, and the bottom graph shows lower abundance miRNAs. Bars represent the mean $\pm$ s.d. number of reads normalized to the total number of reads. Significance was determined with Student’s t-test; bars marked with “*” were significantly different at $P \leq 0.05$. N = 3 replicate samples.

Figure 4. Relative abundance of miRNAs that were over- or underexpressed in diapausing pupae compared to pupae not in diapause. Boxes indicate the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and the median is indicated with a line across the box. N = 7 replicate samples for each pupa type. Boxes labeled with different letters had significantly different medians.

Figure 5. Relative abundance of miRNAs that were over- or underexpressed in nondiapausing pupae from mothers with a diapause history (DH-SD) compared to pupae from mothers with no diapause history (NDH-LD or NDH-SD). Boxes indicate the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and the median is indicated with a line across the box. N = 7 replicate samples for each pupa type. Boxes labeled with different letters had significantly different medians.

Figure 6. Relative abundance of miRNAs that were over- or underexpressed in nondiapausing pupae reared in a long-day rearing environment (NDH-LD) compared to pupae reared in a short-day environment (NDH-SD and DH-SD). Boxes indicate the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and the median is indicated with a line across the box. N = 7 replicate samples for each pupa type. Boxes labeled with different letters had significantly different medians.

Figure 7. Relative abundance of miRNAs that A) were present in a different amount in each of the 3 types of pupae or B) were equivalent in the 3 types of pupae. Boxes indicate the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and the median is indicated with a line across the box. N = 7 replicate samples for each pupa type. Boxes labeled with different letters had significantly different medians.

806 Figure 8. Relative abundance of miRNAs in diapausing pupae and post-diapause pharate adults 24 and
807 48 h after hexane was applied to terminate diapause. Boxes indicate the 25th and 75th percentiles, error
808 bars show the 10th and 90th percentiles, and the median is indicated with a line across the box. N = 7
809 replicate samples for each pupa type. Boxes labeled with different letters had significantly different
810 medians.

811
812 Figure 9. Relative abundance of miRNAs in red-eye pharate adults that had been in diapause compared
813 to ontogenetically matched flies with no diapause history. There was no difference for miR-9c-5p (P =
814 0.593) or miR-13b-3p (P = 0.593). MiR-31a-5p (P = 0.048), miR-125-5p (P = 0.002), miR-190 (DH = 0.005,
815 NDH = 0.003; P = 0.002), and let-7-5p (P = 0.008) were all more abundant in DH flies than NDH flies.
816 Boxes indicate the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and the
817 median is indicated with a line across the box. N = 7 replicate samples for each pupa type. Boxes labeled
818 with different letters had significantly different medians.

Table 1. Summary of mappable reads categorized as known or predicted miRs.

		# Sequences sequences (= reads)	# Unique miRs
Total mappable reads		47,813,193	1,401*
Known miRs			
Group 1	Mapped to known miRNAs in miRBase release 21	81,583	42
Group 1a	Mapped to known <i>S. bullata</i> miRs	0	0
Group 1b	Mapped to other known Arthropods** miRs	81,583	42
Predicted miRs			
Group 2	Mapped to Arthropod mature and precursor miRNA sequences and to the <i>S. bullata</i> genome	85,675	389
Group 2a	Mapped to known Arthropod miR precursors and <i>S. bullata</i> genome – within hairpins	29,164	82
Group 2b	Mapped to known Arthropod miR precursors and <i>S. bullata</i> genome – no hairpins	56,511	307
Group3	Mapped Arthropod mature and precursor sequences in MiRBase, but not to the <i>S. bullata</i> genome***	5,637	866
Group 3a	Mapped to known Arthropod miR precursors and known mature miRs but do not map to <i>S. bullata</i> genome.	4,524	337
Group 3b	Mapped to known Arthropod miR precursors but unmapped to <i>S. bullata</i> genome.	1,113	529
Group 4	Not mapped to Arthropod sequences in MiRBase but did map to <i>S. bullata</i> genome	32,903,797	104
Group 4a****	Not mapped to known Arthropod miRs but are mapped to the <i>S. bullata</i> genome and are within hairpins.	75,258	104
Group 4b	Mapped to the <i>S. bullata</i> genome but extended sequences cannot form hairpins.	32,828,539	---

* Total number of unique miRs is the sum of groups 1b, 2, 3, and 4a

** Arthropod species included in miRBase release 21 were *Drosophila melanogaster* and 11 additional *Drosophila* species, *Tetranychus urticae*, *Rhipicephalus microplus*, *Ixodes scapularis*, *Marsupenaeus japonicas*, *Daphnia pulex*, *Heliconius melpomene*, *Plutella xylostella*, *Locusta migratoria*, *Acyrtosiphon pisum*, *Tribolium castaneum*, *Bombyx mori*, *Manduca sexta*, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Nasonia vitripennis*, *Nasonia giraulti*, *Nasonia longicornis*, *Apis mellifera*, and *Strigamia maritima*

*** Sequences might not have mapped to the *S. bullata* genome draft that was available at the time of sequence analysis.

**** Sequences that fall into this group are potentially novel, *S. bullata* miRNAs

Table 2. KEGG pathways predicted to be collectively* regulated by miRNAs that were differentially regulated in diapausing pupae of *S. bullata*..

KEGG pathway	Number of genes targeted	Number of miRNAs involved	p-value
Mucin type O-Glycan biosynthesis	7	6	1.95E-20
Valine, leucine and isoleucine degradation	17	3	2.37E-12
MAPK signaling pathway - fly	10	6	4.53E-07
Hippo signaling pathway - fly	21	5	2.57E-05
N-Glycan biosynthesis	10	5	2.78E-05
Fatty acid elongation	3	2	6.37E-05
Propanoate metabolism	9	4	0.00019
Biotin metabolism	1	1	0.000305
Phototransduction - fly	11	4	0.001132
Hedgehog signaling pathway	10	4	0.004207
Notch signaling pathway	10	5	0.005148
Phosphatidylinositol signaling system	13	6	0.0117
Fatty acid degradation	7	2	0.017123
Dorso-ventral axis formation	11	5	0.017123
Endocytosis	24	7	0.017123
Glycerophospholipid metabolism	18	9	0.022502
Jak-STAT signaling pathway	8	4	0.032256
Terpenoid backbone biosynthesis	6	3	0.04303
Sphingolipid metabolism	8	7	0.049785

*miRNAs queried include miR-1-3p, miR-289-5p, miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-305-5p, miR-275-3p, and miR-277-3p.

Table 3. Biological processes predicted to be collectively* regulated by miRNAs differentially expressed in diapausing pupae of *S. bullata*.

GO Biological Process	Number of genes targeted	Number of miRNAs involved	P-value
Imaginal disc-derived wing morphogenesis	81	8	3.48e-11
Compound eye development	43	9	2.64e-05
Open tracheal system development	40	8	3.613e-04
Dorsal closure	35	7	5.63e-04
Imaginal disc-derived wing vein specification	20	6	2.27e-03
Mushroom body development	24	8	4.02e-03
Chaeta morphogenesis	19	7	7.24e-03
Cuticle pattern formation	10	5	9.83e-03
Torso signaling pathway	16	8	1.19e-02

*miRNAs queried include miR-1-3p, miR-289-5p, miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-305-5p, miR-275-3p, and miR-277-3p.

Table 4. Mirpath predicted targets of miRNAs that were differentially expressed in diapausing pupae of *S. bullata*.

KEGG Term*	Possible miRNA Regulator	P-value	Predicted Gene Targets	
			Number	Name
DNA replication	miR-289-5p	1.53e-9	3	RPA2, Chrac-14, Mes4
Mismatch repair	miR-289-5p	7.38e-4	1	RPA2
	miR-263a-5p	7.38e-4	1	Msh6
Glycerolipid metabolism	miR-13b-3p	7.83e-9	3	laza, CG6847, CG9886
	miR-289-5p	7.83e-9	3	CG6847, wun, CG34384
	miR-305-5p	6.382e-6	2	CG31140, CG34384
	miR-1-3p	6.382e-6	2	Dgk, CG11426
	miR-275-3p	3.77e-3	1	laza
	miR-263b-5p	3.77e-3	1	CG6753
	miR-277-3p	3.77e-3	1	CG31140
Ether lipid metabolism	miR-289-5p	1.61e-10	3	bbc, wun, CG32699
	miR-13b-3p	1.08e-3	1	laza
	miR-277-3p	1.08e-3	1	CG32699
	miR-1-3p	1.08e-3	1	CG11426
Pyruvate metabolism	miR-289-5p	9.012e-9	3	CG13334, Men-b, CG10970
	miR-277-3p	9.012e-9	3	Mdh1, ACC, CG10932
	miR-13b-3p	3.96e-3	1	AcCoAS
Citrate cycle	miR-289-5p	7.82e-9	3	CG6439, CG5028, ATPCL
	miR-277	7.82e-9	3	Mdh1, ATPCL, Suchb
Riboflavin Metabolism	miR-9a-3p	3.40e-4	1	CG6656
	miR-305-5p	3.40e-4	1	CG9452
	miR-277	3.40e-4	1	Acph-1

*Selected KEGG pathways were enriched in diapausing pupae of *S. crassipalpis* as indicated by microarray analyses of diapause and nondiapause transcriptomes (Ragland et al., 2010).

Table 5. Mirpath predicted targets of miRNAs that were differentially expressed in diapausing pupae of *S. bullata*.

GO Process	Possible miRNA Regulator	P-value	Predicted gene targets	
			number	name
Protein folding GO:0006457	miR-277-3p	1.26e-3	7	Hsc70-5, Hsp60, T-cp1, 14-3-3zeta, CG7048, CG2852, mio
	miR-289-5p	5.80e-3	6	CG32150 ,Trap1, Droj2, 14-3-3zeta, CG4538, jdp
Defense response GO:0006952	miR-289-5p	1.37e-14	13	18w, CG9733, AttC, Sr-CII, crq, Tollo, tub, Pli, dl, srp, Toll-6, CG10433
	miR-13b-3p	1.93e-3	4	CG10345, 18w, PGRP-LC, ninaD
	miR-92b-3p	1.55e-2	3	Imd, Nos, srp
	miR-9c-5p	1.55e-2	3	Traf4, dl, srp
Transaminase activity GO:0008486	miR-277-3p	1.52e-4	2	Got2, CG1673
	miR-289-5p	1.52e-4	2	CG8745, CG1673
	miR-92b-3p	1.76e-2	1	Gfat2
Cellular carbohydrate metabolic process GO:0044262	miR-277-3p	3.83e-5	2	Mdh1, ATPCL
	miR-289-5p	3.83e-5	2	CG13334, ATPCL
Response to stimulus GO:0050896	miR-289-5p	4.2e-25	17	sens , Or22a, Rh7, Fmr1, R, Arr1, CdsA, norpA, mys, faf, Galpha49B, B-H1, Rh3, nonA, poe, gl, aos
	miR-92b-3p	4.93e-7	6	Or47a, Obp57d, Obp57c, Arr1, CdsA, aos
	miR-9c-5p	1.08e-5	5	sens, trpgamma, norpA, rdgC, gl
Unfolded protein binding GO:0051082	miR-277-3p	1.45e-4	5	Hsc70-5, Hsp60, T-cp1, CG7048, mio
	miR-289-5p	1.54e-3	4	Trap1, Droj2, CG4538, jdp
Chaperonin containing T-complex GO:0005832	miR-277-3p	3.61e-3	1	T-cp1
Nuclear pre-replicative process GO:0005656	miR-289	1.08e-8	2	rec, Cdc6
	miR-13b	1.65e-4	1	Cdc6

*Selected KEGG pathways were enriched in diapausing pupae of *S. crassipalpis*, as indicated by microarray analyses of diapause and nondiapause transcriptomes (Ragland et al., 2010).

Table 6. Genes known to be differentially expressed in diapausing *S. crassipalpis* that are putative targets of miRNAs that were downregulated in diapausing *S. bullata*.

Downregulated miRNAs	Upregulated Transcripts ¹	Upregulated proteins ^{2,3}
miR-9c-5p	<i>Pepck, Giiispla2, Gstd1, CG10570, Dnaj, CG14509, gasp, obst-A</i>	CG6711 ² , Pkd2 ² , Hsp70Bc ³
miR-13b-3p	<i>Scylla, CG10570, Pink1, ckd, CG10924, CG31915, Ald</i>	CG10431 ² , RET ² , CG15249 ² , Adat ² , SWS ²
miR-31a-5p	<i>CG31883, Cyp12a4, Gasp, PGRP-SD, InR, bmm</i>	RET ² , Adat ²
miR-92b-3p	<i>CG11299, Hsp60, zasp66 (CG6416), cactus, sec5, InR, Hsc70-5, Cyp6g1, vir-1</i>	Catalase1 ² , Su(dx) ² , Fabp ³
miR-275-3p	<i>AcCoAs, Pink1, Men, Act57B</i>	RET ² , CG6711 ²
miR-276a-5p	<i>CG6188, Hsp70Bbb, kraken, sec5, AcCoAs, Pgm, GstD1, CG10924, CG31883, Cyp12a4, parp, Hsf, Trap1, inaD</i>	Argk ² , Su(dx) ² , Fabp ³
miR-277-3p	<i>Hsp27, CG15609, Hsc70-5, Hsp60, Hop, Hsp67Ba, pdh, CG31075</i>	RET ²
miR-305-5p	<i>AcCoAs, InR, bmm, Dnaj, CG31915, Hsp70Bbb, Hsc70-1, ckd, Defense, Hsc70-2, Ald</i>	Catalase1 ² , Argk ² , CG10431 ² , CG6711 ² , CG15249 ² , Pkd2 ² , Adat ² , SWS ² , Hsp70Bc ³ , Fabp ³

¹Ragland et al., 2010

²Pavlidis et al., 2011

³Li et al., 2009

Table 7. Pathways that may be targeted by miRNAs* that were differentially expressed in pupae that did not enter diapause due to the maternal effect that blocks diapause entry.

KEGG pathway	No. of genes targeted	No. of miRNAs involved	p-value
Mucin type O-Glycan biosynthesis	6	3	8.05E-17
Valine, leucine and isoleucine degradation	16	2	1.41E-11
MAPK signaling pathway - fly	8	3	2.87E-06
Biotin metabolism	1	1	1.42E-04
Hippo signaling pathway - fly	19	2	1.42E-04
Phototransduction - fly	11	3	1.58E-04
N-Glycan biosynthesis	8	4	3.82E-04
Hedgehog signaling pathway	10	3	1.21E-03
Endocytosis	24	5	2.05E-03
Propanoate metabolism	7	2	5.20E-03
Dorso-ventral axis formation	11	4	5.99E-03
Notch signaling pathway	9	3	6.87E-03
Phosphatidylinositol signaling system	11	4	1.08E-02
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	3	3	1.439E-02
Fatty acid elongation	2	1	0.021628051
Valine, leucine and isoleucine biosynthesis	1	2	0.047849606

* miRNAs queried included let-7-5p, miR-100-5p, miR-125-5p, miR-263a-5p, miR-9c-5p, miR-289-5p, miR-277-3p and miR-92b-3p.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Relative miRNA Abundance

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9