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# Transcriptional evidence for small RNA regulation of pupal diapause in the flesh fly, *Sarcophaga bullata*

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ABSTRACT

Understanding the molecular basis of diapause, a phenotypically plastic, alternative developmental pathway, is key to predicting the seasonal distribution of economically and medically important insect species. Small regulatory RNAs, including piwi-related RNAs, small-interfering RNAs, and miRNAs, represent one type of epigenetic process that can alter the phenotype of organisms independent of changes in genome sequence. We hypothesize that small RNAs regulate pupal diapause and a maternal block of diapause in the flesh fly *Sarcophaga bullata*. We assessed the relative abundance of eight genes related to small RNA biogenesis and function using qRT-PCR in pre-diapause and diapause stages compared to their non-diapause counterparts. Elevated mRNA expression of *piwi* and *spindle-E*, as well as *argonaute2* and *r2d2*, in photosensitive 1st instar larvae reared in diapause-inducing conditions indicate involvement of the piwi-associated RNA and small-interfering RNA pathways, respectively, in programming the switch from direct development to a developmental pathway that includes diapause. Two genes, related to the microRNA pathway, *argonaute1* and *loquacious*, are upregulated during pupal diapause, suggesting a role for this pathway in maintaining diapause. Substantial reduction in transcript abundance of small RNA-related genes in photosensitive 1st instar larvae from mothers with a diapause history compared to those from mothers with no diapause history also suggest a role for small RNA pathways in regulating a diapause maternal effect in *S. bullata*. Together, the results point to a role for small RNAs in regulating the developmental trajectory in this species.



## 1. Introduction

The developmental program of the flesh fly, *Sarcophaga bullata*, can be switched from "direct-development" to "diapause-destined" when embryos and 1st instar larvae are exposed to short day lengths (<13.5 h of light per 24 h; Denlinger, 1971, 1972). The ability to switch on the diapause program is advantageous for these flies, and other insects, because this dormant state provides a means to escape seasonal periods of unfavorable conditions and to coordinate periods of growth and development with optimal conditions. While the adaptive significance of entering diapause is clear, the mechanisms that regulate diapause are still not well understood. Previous research has characterized significant changes in the transcriptomes of pre-diapause (Reynolds and Hand, 2009; Poelchau et al., 2011), diapause (Denlinger, 2002; Ragland et al., 2010; Poelchau et al., 2013), and post-diapause (Emerson et al., 2010; Ragland et al., 2011) insects. However, the mechanisms that regulate the observed changes in transcription remain a conspicuous knowledge gap.

The relatively recent discovery of small non-coding RNAs (sRNAs) provide new opportunities for uncovering the molecular basis of diapause. sRNAs represent one type of epigenetic process that can alter the phenotype of an organism independent of changes in DNA sequence. sRNAs trigger gene silencing pathways and are increasingly recognized as important regulators of diverse biological functions including cell-cycle regulation and cell proliferation (Sun et al., 2008), developmental timing (Bethke et al., 2009; Hammell et al., 2009; Resnick et al., 2010), metabolism (Xu et al., 2003), stress resistance (Teleman and Cohen, 2006; Flynt et al., 2009; Zhang et al., 2011), fecundity (Klattenhoff et al., 2007), immune function (Kemp et al., 2013) and other processes known to be altered before, during, and after diapause. Moreover, sRNAs contribute to the maintenance of cellular homeostasis by providing a link between environmental signals and necessary changes in physiology and/or biochemistry. Specifically, sRNAs are

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important mediators between nutrient availability and energy homeostasis through their regulation of the insulin signaling pathway in *Drosophila melanogaster* (Teleman and Cohen, 2006; Lim et al., 2011), and they also coordinate the switch between direct development and dauer formation in response to high temperatures, low food ability, and the presence of dauer hormone in *Caenorhabditis elegans* (Hall et al., 2010, 2013). Finally, sRNAs coordinate hormonal activity with changes in growth and development (Boulan et al., 2013; Bethke et al., 2009), which are both integral features of the diapause program. Given that sRNAs are associated with multiple processes that are essential for diapause, we predict that they have a significant role in regulating the initiation, maintenance, and termination of diapause.

sRNAs are subdivided into three classes based on their size, origin, structure, localization, and mode of action. MicroRNAs (miRNAs) and small-interfering RNAs (siRNAs) are 20–25 nucleotides long and are processed from double-stranded regions of long, non-coding RNAs; they are found in somatic cells throughout the body. Both miRNAs and siRNAs originate from double-stranded RNAs and are initially processed by dsRNA specific nucleases called Dicers that are assisted by dsRNA binding proteins. In *Drosophila*, pre-miRNAs are processed by Dicer1 and its partner Loquacious (Loqs) while pre-siRNAs are processed through the combined action of Dicer2 and R2D2 (Siomi and Siomi, 2009; Tomari et al., 2007). The resulting sRNAs are differentially sorted to an RNA-induced silencing complex (RISC) based on fundamental differences in the structures of miRNAs and siRNAs. The catalytic center of each RISC is a member of the Argonaute protein family. miRNAs are sorted to RISCs containing Argonaute 1 (Ago1); siRNAs associate with RISCs containing Argonaute 2 (Ago2). Once associated with the RISC, miRNAs generally block translation of their target mRNA while siRNAs promote transcript degradation.

The piRNA pathway is limited to gonadal tissue, where it is found in both somatic and germline cells. piRNAs (22–31 nt) originate from single-stranded RNAs including transposable elements (see reviews by Juliano et al., 2011; Thompson and Lin, 2009). The initial steps of piRNA biogenesis are not understood, but an RNA-helicase, Spindle-E (Spin-E), is required (Kennerdell et al., 2002; Olivier et al., 2010; Handler et al., 2013). Mature piRNAs are loaded into a RISC that contains one of three Piwi-class Argonaute proteins: Piwi, Aubergine (Aub), or Argonaute 3 (Ago3). The Argonaute protein in the pi-RISC specifies the fate and function of the piRNA. Piwi-piRNA complexes, found in both somatic and germline cells, are transported into the nucleus where they regulate gene expression. Aubergine-piRNA and Ago3-piRNA complexes are limited to germline cells and remain in the cytoplasm where they regulate expression of transposable elements (Handler et al., 2013).

To assess the potential role of sRNAs in regulating pupal diapause, we used quantitative RT-PCR (qRT-PCR) to measure RNA expression of genes that encode core components of miRNA, siRNA, and piRNA pathways in 1st instar larvae and phanerocephalic pupae (the stage of diapause) in *S. bullata*. Components of all three pathways have been well characterized in *D. melanogaster* (Kolaczkowski et al., 2011), *Tribolium* spp. (Tomoyasu et al., 2008), and *Bombyx mori* (Kolliopoulou and Swevers, 2013), and they are generally well conserved among insect species.

*S. bullata* is an ideal species for addressing questions about sRNA regulation of diapause, in part because the physiological (Denlinger et al., 1972), biochemical (Michaud and Denlinger, 2007; Rinehart et al., 2007) and transcriptional changes associated with diapause have been well characterized for this and a closely related species (Hahn et al., 2009; Rinehart et al., 2010; Ragland et al., 2010). *S. bullata* is an especially interesting model for studying sRNA regulation of diapause because a maternal effect in this species completely blocks diapause entry in progeny of females that experienced diapause themselves (Henrich and Denlinger, 1982; Rockey et al., 1989; Denlinger, 1998). Thus, our study not only provides novel information about regulation of diapause but also provides insight on the relative influence of maternal history and rearing environment on the switch between two alternative developmental pathways.

### 1.1. Methods and materials

#### 1.1.1. Insect rearing

*S. bullata* were from our established laboratory colony and were reared as previously described (Denlinger, 1972). Briefly, adults were maintained under either short-day conditions that induce diapause in their progeny (25 °C, 8 h photophase) or long-day conditions to prevent diapause induction (25 °C, 16 h photophase). 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 and then provided 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, and aspen SaniChips (Harlan, Indianapolis, IN, USA) were provided as a pupariation site. All larvae were reared at 18 °C; pre-diapause larvae were maintained under a short-day photoperiod and non-diapause larvae were maintained under a long-day photoperiod.

Diapause was terminated pharmacologically by removing the anterior cap of the puparium and applying 5 μl of hexane to the exposed head (Denlinger et al., 1980). Hexane-treated flies were kept at 25 °C until adult emergence and were then transferred to either short-day or long-day conditions. Larval progeny of flies with a diapause history (DH) were reared under the same light regime as their parents (short- or long-day, respectively).

### 1.2. Transcript profiling

A comparative approach was used to identify putative *S. bullata* homologs of *D. melanogaster* genes that encode components of the three small RNA pathways. Nucleotide sequences of *D. melanogaster* genes, identified from Flybase (http://flybase.org/), were used to perform tblastx searches (Altschul et al., 1990) against a *Sarcophaga crassipalpis* database (Hahn et al., 2009). To verify identity of the *S. crassipalpis* EST and to confirm significant homology between *S. crassipalpis* and *S. bullata*, we performed PCR using primers designed from the *S. crassipalpis* nucleotide sequence and a cDNA template synthesized from total RNA isolated from *S. bullata*. The resulting PCR product was cloned using the StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Plasmids were sequenced, and sequence identity was confirmed using blastx (Altschul et al., 1990). Results of the BLAST search are given in Table 1.

Relative transcript abundance was assessed in photosensitive 1st instar larvae and in phanerocephalic pupae, the stage of diapause, from mothers with or without a history of diapause (Fig. 1). Total RNA was isolated from larvae or pupae using a Purlink RNA Isolation kit (Life Technologies) according to an alternate protocol that uses TRIzol® for the initial step. PureLink DNase was used to remove genomic DNA.

Concentration of each RNA sample was assessed using a Nano Drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's directions; equal amounts of total RNA were used in each reaction. Two independent reactions were carried out for each replicate sample and then pooled.

Relative mRNA abundance of selected genes of interest was measured using an iQ5™ Multicolor Real-time PCR Detection