Current Research in Insect Science 6 (2024) 100099

Contents lists available at ScienceDirect

Current Research in Insect Science

journal homepage: www.elsevier.com/locate/cris

Differentially expressed microRNAs in brains of adult females may regulate the maternal block of diapause in *Sarcophaga bullata*

Julie A. Reynolds [a,*], Emma M. Waight [a,b]

[a] Department of Evolution, Ecology, and Organismal Biolog, The Ohio State University, Columbus, OH 43210, United States
[b] Hablitz/Nedergaard Lab, Center for Translational Neuromedicine, University of Rochester Medical Center. Rochester, NY 14642, United States

ARTICLE INFO

Keywords:
Insects
Epigenetics
Anticipatory transgenerational effects
Dormancy
Circadian clock
Seasonal response

ABSTRACT

The maternal regulation of diapause is one type of phenotypic plasticity where the experience of the mother leads to changes in the phenotype of her offspring that impact how well-suited they will be to their future environment. *Sarcophaga bullata* females with a diapause history produce offspring that cannot enter diapause even if they are reared in a diapause inducing environment. Accumulating evidence suggests that microRNAs regulate diapause and, possibly, maternal regulation of diapause. We found significant differences in the abundances of several microRNAs (miR-125–5p, miR-124–3p, miR-31–5p, and miR-277–3p) in brains dissected from adult female *S. bullata* that had experienced diapause compared to females with no diapause history. We also found moderate differences in the mRNA expression of the circadian-clock related genes, *clock, clockwork orange,* and *period*. MiR-124–3p and miR-31–5p are part of a gene network that includes these circadian clock-related genes. Taken together our results suggest the maternal block of diapause in *S. bullata* is regulated, at least in part, by a network that includes microRNAs and the circadian clock.

Introduction

Maternal effects, also referred to as anticipatory parental effects or transgenerational effects (Kronholm 2022; Marshall and Uller, 2007) are a type of phenotypic plasticity that occurs when the mother detects environmental cues that forecast changes in the environment and, consequently, alteres her reproductive strategy to improve her offsprings' ability to survive. One example is the maternal regulation of diapause in insects. Mothers sense changes in photoperiod or temperature that are indicative of a future decline in habitat suitability (e.g., limited availability of high-quality food and/or water supply). These signals initiate an alternative developmental program in their offspring that includes a period of dormancy known as diapause.

Diapause is an endogenously regulated dormant state that occurs at a genetically predetermined stage of the life cycle. Entering diapause provides insects a mechanism to deal with seasons defined by extreme temperatures, limited water, and/or insufficient sources of nutrition. The diapause program is characterized by developmental arrest, metabolic depression, and increased tolerance of environmental insults. A key feature of diapause is that it is initiated well before the environment becomes inhospitable. For a number of insects, including crickets, wasps, aphids, moths, beetles, flies, and mosquitoes (Henrich and Denlinger, 1982; Lee and Duvall, 2022; Mousseau and Fox, 1998; Mukai et al., 2022; Reznik and Samartsev, 2015; Tougeron et al., 2018) diapause is regulated by a maternal effect. The change in seasons (i.e., the signal to enter diapause) is perceived by the mother and is translated into a signal that switches the developmental trajectory of her offspring so that they enter diapause instead of developing directly to the next stage of the lifecycle. Most insects that exhibit maternal regulation promote diapause in their offspring. However, there are insects, including the flesh fly, *Sarcophaga bullata*, that have a maternal effect that blocks diapause entry rather than promoting it (Henrich and Denlinger, 1982). Prohibiting diapause entry in flies that are active during the early spring provides an additional mechanism for synchronizing the developmental timing with resource abundance and ensures the population has opportunity to take advantage of an environment can support growth and reproduction.

S. bullata enter diapause during the pupal stage if they experience short daylengths (less than 12 h of daylight per 24 h) and cool temperatures as photosensitive embryos or 1st instar larvae (Denlinger, 2022). However, females that experienced diapause themselves (i.e., have a diapause history) or were reared under a short-day photoperiod,

* Corresponding author.
E-mail address: reynolds.473@osu.edu (J.A. Reynolds).

https://doi.org/10.1016/j.cris.2024.100099
Received 11 January 2024; Received in revised form 8 September 2024; Accepted 25 September 2024
Available online 26 September 2024

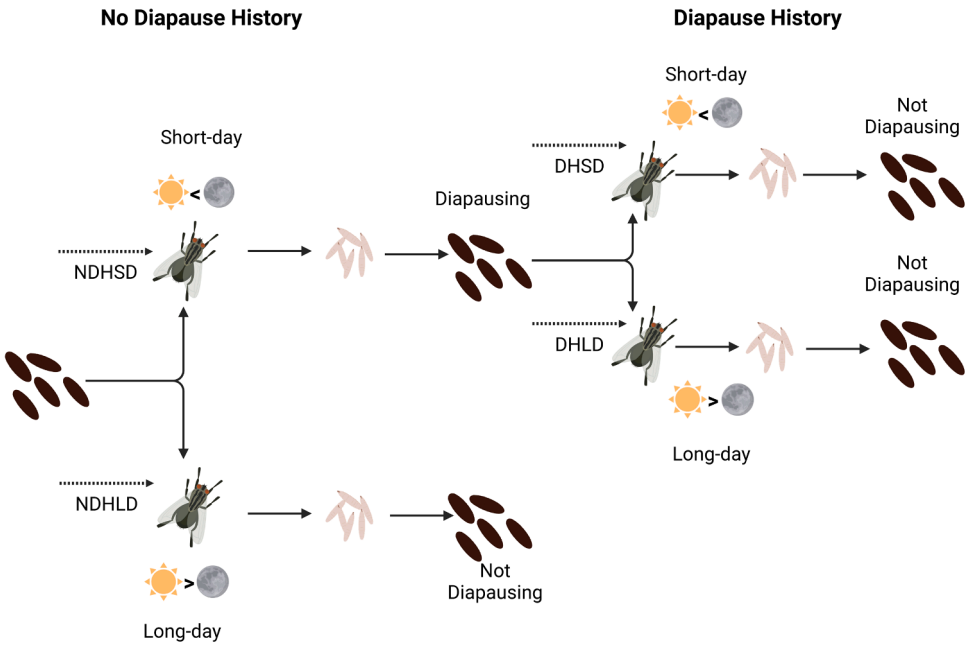

Fig. 1. Experimental design and sampling scheme. Brains dissected from female adult flies, indicated with dashed arrows, were used for all experiments. Flies from the established colony with no diapause history were placed in diapause-inducing short-day (SD) conditions or diapause averting long-day (LD) conditions during the pupal stage. Flies in long-day conditions were haphazardly collected 3 d after adult emergence, and females were assigned to the no diapause-history, long-day (NDHLD) group. Flies in short-day conditions were haphazardly collected, and females were assigned to the no diapause-history, short-day (NDHSD) group. The remaining flies in short-day conditions produced offspring that entered diapause. After diapause termination, flies were placed in short-day or long-day conditions. Adult females were collected for the DHSD and DHLD groups 3 d after eclosion.

produce offspring that cannot enter diapause even if they are reared in diapause inducing conditions (Henrich and Denlinger, 1982). The mechanism that regulates this maternal block of diapause is not well understood, but prior research suggests that it involves a signaling pathway that includes γ-Aminobutyric acid (GABA), and possibly other neurotransmitters. This pathway is active during the first 3 d after adult eclosion, and it regulates expression of the maternal effect by communicating between a female's brain and her ovaries (Rockey et al., 1989; Webb and Denlinger, 1998). More recent research suggests this maternal block of diapause is also regulated to some degree by epigenetic factors including histone modifications and small noncoding RNAs (Reynolds et al., 2013, 2016 and 2017).

The current study investigates differences in the brains of *S. bullata* females that experienced diapause (i.e., have a diapause history) compared to females that do not have a diapause history. We also considered the molecular underpinnings of photoperiod in the expression of the maternal effect by comparing the brains of females reared under a diapause-inducing short-day photoperiod to brains of females reared in a diapause-averting, long-day environment. We evaluated the abundances of candidate microRNAs (i.e., small noncoding RNAs that post-transcriptionally regulate gene expression) to test the hypothesis that differentially regulated microRNAs are involved in the maternal block of diapause). We also evaluated the abundances of *clock, period, timeless,* and other genes related to circadian timing to test the hypothesis that the circadian clock participates in the expression of the maternal effect. We found that miR-125–5p, miR-31–5p, miR-124–3p were differentially regulated in a way that suggests they regulate the maternal effect. Transcriptional regulation of clock-related genes does not appear to be associated with expression of the maternal effect because significant differences in transcript abundance were linked to differences in photoperiod rather than diapause history.

Methods

Colony maintenance

Sarcophaga bullata from an established colony were maintained as previously described (Reynolds et al., 2013; Denlinger, 1972). Briefly, all life stages were kept at 25 °C in an environment with a long-day photoperiod (16 h of light per 24 h). Adults had unlimited access to water and sucrose. Beef liver was provided as a protein source. Fresh liver was provided for larviposition 11–13 d post-eclosion. Larvae were provided enough liver to complete this stage of development, and aspen SaniChips (Harlan, Indianapolis, IN, USA) were provided as a pupation site.

Experiment design and sample preparation

Pupae with no diapause history (NDH) were haphazardly divided into two groups. One group was transferred to a diapause-inducing environment with a short-day (SD) photoperiod (8 h of light per 24 h). The second group was reared in a diapause-averting environment with a long-day photoperiod (LD). Three days after the adults emerged, some females from each group were collected for brain dissections and RNA isolation. These females made up the nondiapause-history, long-day (NDHLD) and nondiapause- history, short-day (NDHSD) samples (Fig. 1).

The remaining short-day flies were allowed to mate and produce offspring. Their progeny entered diapause at the pupal stage. Approximately 40 d after pupariation diapause was terminated using hexane a previously described. (Denlinger et al., 1980; Reynolds et al., 2017). Briefly, the anterior cap of the puparium was removed, and 5 μl hexane was applied to the exposed head. Following hexane treatment, flies were transferred to a 25 °C chamber under a short-day or long-day photoperiod. They were kept in these conditions until adults emerged (Fig. 1). Three days post-emergence, females from each group were collected for further studies. These females made up the diapause-history, long-day

Table 1
Circadian clock-related genes in *S. bullata*.

Gene name	*S bullata* ID	Sequence ID of Closest Match	Closest relative	e-value	% Identity
period	TMW51428.1	AB080236.3	*S. bullata*	0	98%
timeless	TMW43778.1	AB080235.2	*S. bullata*	0	88%
clock	TMW51775	XP_037820492.1	*Lucilia cuprina*	0	83%
clockwork orange	TMW53451.1	KAI8130879	L. *cuprina*	0	82%
Pdp1	TMW51764	AAF15511.1	*D. melanogaster*	3e-132	97%
vrille	TMW49993.1	NP_001285623	*D. melanogaster*	5e-73	73%

((DHLD) and diapause-history, short-day (DHSD) samples.

Brains were dissected from females 3 d after adult emergence. We chose this age because prior research showed this interval is critical for expression of the maternal effect (Rockey et al., 1989). All dissections were performed 5 to 6 h after "lights-on" to control for differences in diurnal expression profiles. Dissections were performed on fresh, unfrozen tissue in sterile insect saline. Brains were put directly into a homogenization buffer for further processing. Three brains were included in each sample, and we used 5–6 replicate samples for each group.

Total RNA was extracted from dissected brains using the miRVana™ RNA Isolation kit (Life Technologies, Carlsbad, California, USA) according to the manufacturer's direction. The RNA yield (μg/μl) was quantified, and the purity was evaluated using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized using the miScript Reverse Transcription kit (Qiagen, Germantown MD, USA) according to the manufacturer's instructions for HiFlex buffer, which allows measurement of both microRNAs and mRNA transcripts in the same sample. One microgram of total RNA was used for each cDNA synthesis reaction.

Quantitative reverse-transcript PCR

MicroRNA and mRNA sequences are from previously published studies. *S. bullata* microRNAs were originally identified in Reynolds et al., 2017. Sequences are available in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nih.gov/geo/) under accession number GSE94376. Sequences for circadian-clock related genes (Table 1) came from the *S. bullata* genome (Martinson et al., 2019) and are available under NCBI Bioproject number PRJNA476317. Sequences for *period* and *clock* were originally published by Goto et al., 2006.

We measured the relative abundance of candidate microRNAs and mRNAs using an iQ5™ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative abundance of candidate microRNAs was measured using miScript Assays (Qiagen, Valencia, CA, USA) following the developer's recommendations. The abundances of candidate mRNAs were measured using Luna® Universal qPCR Master Mix (New England BioLabs, Ipswich, MA, USA) and primers designed using PrimerQuest software (Integrated DNA Technology, Coralville, IA, USA). Primers for microRNAs and mRNAs conformed to MIQE standards (Bustin et al., 2010); the efficiency and R^2 values for each primer pair are shown in Supplemental Tables S1 and S2. Cycling parameters for miScript assays were 94 °C for 15 m followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Cycling parameters for mRNA assays were 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. Melt curve analysis and gel electrophoresis confirmed that only one product was formed under these conditions.

Relative abundance of microRNAs or mRNAs was calculated in replicate samples using modified $2^{-\Delta Ct}$ (Reynolds et al., 2013). Briefly, we found the average threshold cycle (Ct) of three technical replicates (i.e., three wells of a 96-well plate) for each replicate sample. We normalized the value found for each replicate sample by subtracting the geometric mean of the Ct values measured for Rp49 and Histone H3. These two reference genes were selected because their Ct values did not show significant differences across all the samples (Supplementary Fig. S1). qRT-PCR data and calculations are supplied in Supplementary File 1.

One-way ANOVA was used to compare the relative abundances of the four groups (MiniTab, State College, PA, USA). Tukey's was used post-hoc for pair-wise comparisons between groups. Graphs were created using GraphPad Prism 9.5.1.

Results and discussion

Possible role for microRNAs in the expression of the maternal effect

A maternal effect in *S. bullata*, prohibits diapause entry in the offspring of females that experienced diapause (i.e., has a diapause history) even if they are reared in a diapause-inducing environment (Henrich and Denlinger, 1982). We know from prior research that expression of this maternal effect involves a regulatory signal that is communicated from the brain to the ovaries during the first three days after adults eclose (Rockey et al., 1989). Neurotransmitters, including GABA, octopamine, and pilocarpine (i.e., an acetylcholine agonist) are involved in some capacity. Injecting GABA into females with no diapause history mimics the maternal effect and significantly reduces diapause incidence in the progeny of treated females even under diapause-inducing conditions. Injecting picrotoxin, octopamine or pilocarpine into females partly reverses the maternal effect and leads to a modest increase in diapause incidence compared to control flies (Webb and Denlinger, 1998). More recent studies suggest that expression of the maternal effect involves regulatory epigenetic processes (e.g., histone modifications and gene silencing by small noncoding RNAs) that mediate diapause initiation in photosensitive 1st instar larvae (Reynolds et al., 2013; 2016; 2017).

The current study provides additional information needed to uncover the mechanisms underpinning the maternal effect in *S. bullata*. We used qPCR to evaluate the relative abundances of candidate microRNAs and genes related to the circadian clock in brains dissected from adult female flies 3 d after they eclosed. We compared these relative abundances between the four experimental groups — nondiapause-history, long-day (DHLD); nondiapause-history, short-day (NDHSD); diapause-history, long-day (DHLD); and diapause-history, short-day (DHSD). NDHLD and NDHSD females had not previously experienced diapause (i.e., had a nondiapause history) and were kept in diapause-averting (LD) conditions or diapause-inducing (SD), respectively. DHLD and DHSD females had a diapause history and were kept in SD or LD conditions (Fig. 1). We found that miR-31–5p, miR-124–3p, miR-277–3p, let-7–5p, and miR-125–5p were differentially regulated in at least one of the four groups. We also found significant differences in the mRNA expression of the circadian clock-related genes *clock, period, timeless, clockwork orange,* and *vrille.* Together these data provide new information about possible interactions between microRNAs, the circadian clock, and expression of the maternal effect in *S. bullata*.

The microRNAs we evaluated (miR-9c-5p, miR-31a-5p, miR-124–3p, miR-263–3p, miR-275–3p, miR-277–3p, miR-289–5p, let-7–5p, miR-100–5p, and miR-125–5p) were selected because 1) their relative abundances in diapausing and nondiapausing pupae of *S. bullata* suggest they are involved in the expression of the maternal effect (Reynolds et al., 2017) and/or 2) these miRNAs are part of diapause-relevant regulatory networks in other insects. In many cases these microRNAs

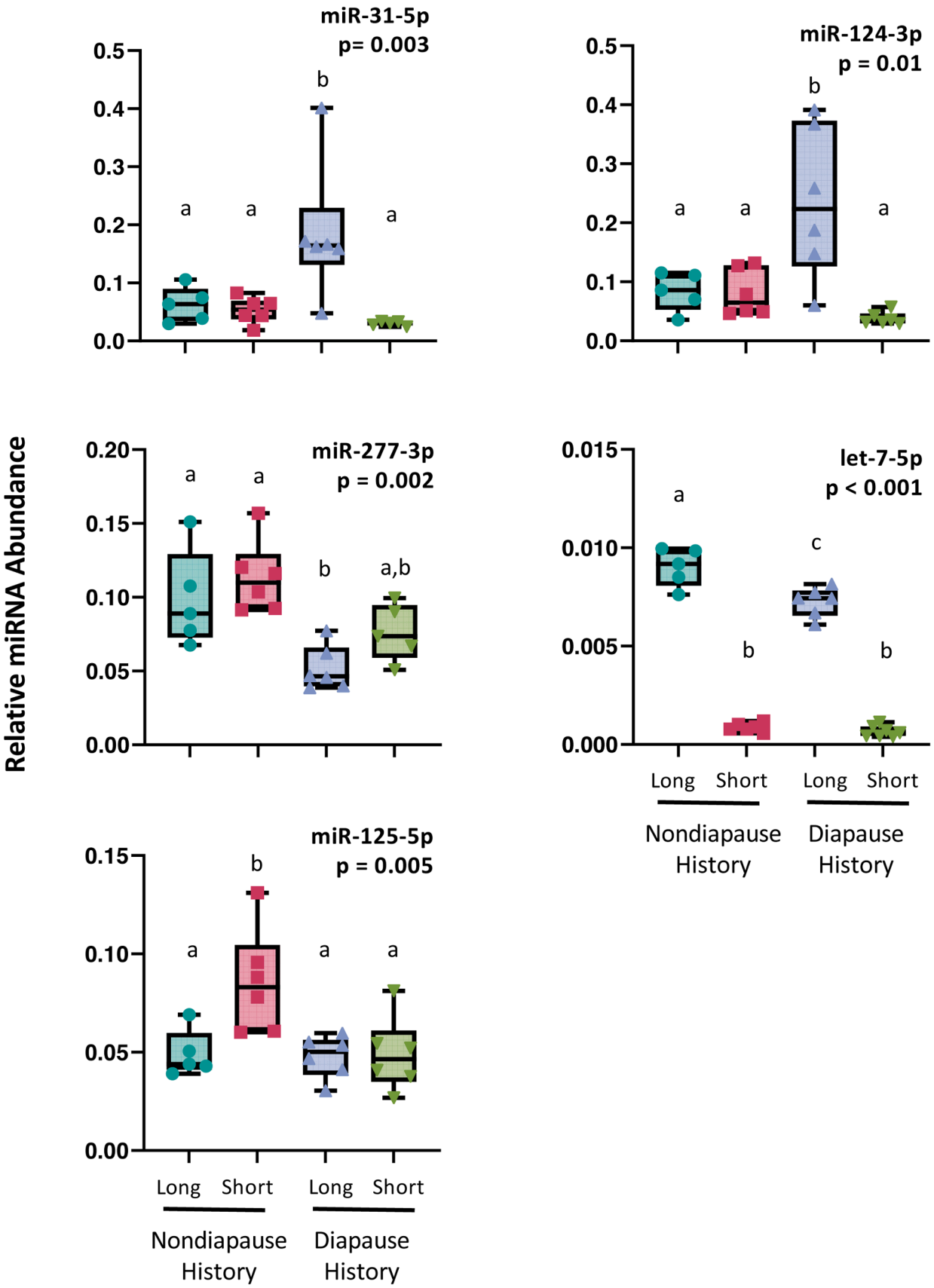

Fig. 2. Relative abundances of candidate microRNAs in brains dissected from adult females of *S. bullata.* Box and whisker plots, points represent independent biological replicates. Boxes that do not share a letter are significantly different from each other. $N = 5–7$ replicate samples with 3 brains each.

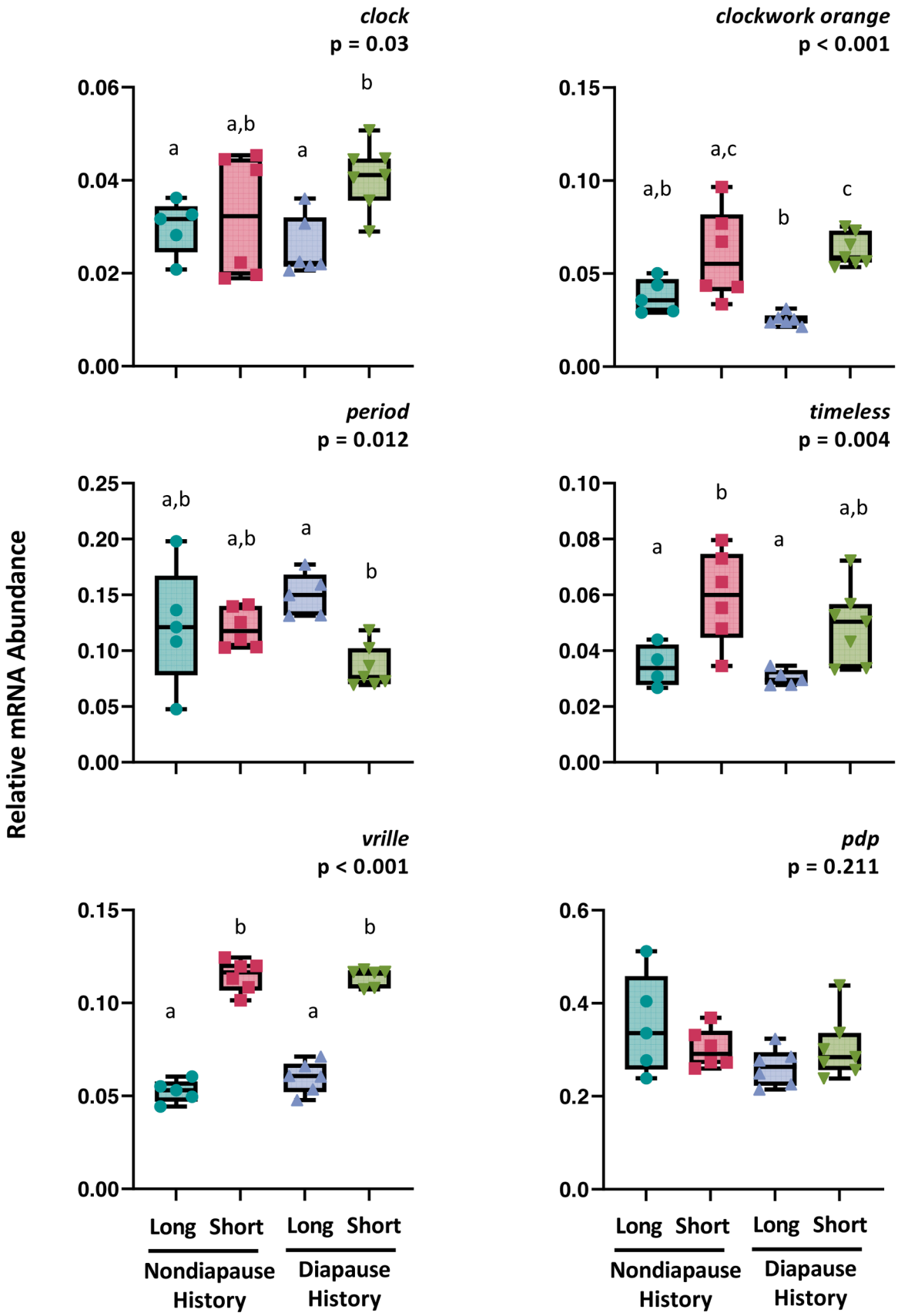

Fig. 3. Relative abundances of circadian clock-related genes in brains dissected from adult females of *S. bullata.* Points on the box and whisker plots represent independent biological replicates. Boxes that do not share a letter are significantly different from each other. $N = 5–7$ replicate samples with 3 brains per sample.

are part of networks that also include circadian clock-related genes and endocrine signaling pathways as we discuss below.

We found significant differences ($p \leq 0.05$) for let-7–5p, miR-31a-5p, miR-124–5p, miR-277–3p, and miR-125–5p (Fig. 2). Let-7–5p was elevated 8-fold the brains of females from the LD environment compared to females kept under a SD photoperiod (Fig. 2). Diapause history only had a modest influence on let-7 abundance for LD flies with an ~ 1.3-fold reduction in the brains of DH females. In *D. melanogaster* let-7 is required for normal circadian rhythms and is part of a feedback loop that also includes the circadian regulatory cycle and ecdysone synthesis pathway (Chen et al., 2014).

MiR-31 and miR-124 were both elevated ~ 4-fold in the brains of diapause-history, long-day (DHLD) females relative to the other three groups (Fig 2b and 2c, respectively). In *S. bullata* the maternal effect is maintained in every succeeding generation if females remain in an environment with a short-day photoperiod (Henrich and Denlinger, 1982). Rearing one full generation under a long-day photoperiod "erases" the maternal effect and restores the potential to enter diapause in the next generation (Henrich and Denlinger, 1982). One hypothesis is that overexpression of miR-31–5p and miR-124–3p in diapause-history, long-day (DHLD) brains is part of the process that "erases" (i.e., removes) the maternal effect. In general, miRNAs mediate the switch between alternative developmental pathways (Reynolds, 2024), and it is possible that miR-31–5p and miR-124–3p may alter one or more signaling pathways that are necessary for the maternal effect to be expressed.

MiR-31–5p is part of a regulatory network in humans that includes the protein Clock and the MAPK/ERK signaling pathway (Yu et al., 2021). We do not know if miR-31–5p, MAPK/EKR and Clock interact in insects. However, miR-31a-5p is downregulated in *S. bullata* when diapause is terminated with hexane (Reynolds et al., 2017), and MAPK/EKR signaling is activated during diapause termination in *Sarcophaga crassipalpis*, a close relative of *S. bullata* (Fujiwara and Denlinger, 2007). In addition, miR-31–5p is part of a regulatory network in brains of adult *Drosophila melanogaster* that mediates the formation of glia and may also regulate diapause (Foo, 2017). A recent study in *D. melanogaster* showed that some glia respond to changes in ecdysone titers through an unknown process that leads to changes in the sleep-awake cycles and in lipid metabolism (Li et al., 2023). Together these results provide some evidence that suggests an interaction between miR-31–5p, MAPK/EKR, and the circadian clock could work together to regulate diapause.

MiR-124–3p regulates nervous system development in *D. melanogaster* (Wang et al., 2014). It also has a rhythmic expression pattern, and it regulates circadian output (i.e., locomotor activity) without impacting the central clock (Zhang et al., 2016; Anna and Kannan, 2021). miR-124–3p targets Bone morphogenic protein (BMP) which, in turn, regulates expression of production of *clock* in the pigment dispersing factor (pdf) neurons in brains of adult *D. melanogaster* (Beckwith et al. 2013). miRNA-124–3p has not been evaluated in diapausing pupae of *S. bullata*, but its abundance is lower in pre-diapause/early diapause adults of *Culex pipiens* which suggests it is part of the diapause initiation network in these mosquitoes (Meuti et al., 2018). In addition, miR-124–3p targets the gene *Pigment dispersing factor* (*pdf*) (Zhang et al., 2016) which regulates diapause in *Pyrrhocoris apterus* and *Culex pipiens* (Kotwica-Rolinska et al., 2022; Meuti et al., 2015). Additional studies on function of miRNA-124–3p in *S. bullata* are needed to determine its role in the diapause maternal effect.

The most intriguing differences were found for miR-277–3p and miR-125–5p (Fig 2). miR-277–3p abundance was reduced 1.3 to 2-fold in the brains of females with a diapause history compared to females with no diapause history, regardless of their rearing environment. We predict that miR-277–3p regulates the maternal effect in *S. bullata* as part of a network that includes ecdysone and neurotransmitters such as dopamine, octopamine, and serotonin. In *Bombyx mori* miR-277–3p is regulated by 20-hydroxy ecdysone (20-HE; Jin et al., 2020). In *Helicoverpa armigera* miR-277–3p targets *Dopa decarboxylase*, an enzyme in the dopamine and serotonin biosynthesis pathway (Shen et al., 2020). As with most microRNAs, increasing miR-277–3p reduces expression of *Dopa decarboxylase* at the mRNA and proteins levels. Dopamine, serotonin, octopamine, and GABA regulate reproductive dormancy in *D. melanogaster* (Andreatta et al., 2018), and although ovarian arrest is not a feature of diapause in *S. bullata*, GABA and octopamine have been implicated in the expression of the maternal effect (Webb and Denlinger, 1998). Additional studies will further explore the relationships between miR-277–3p, ecdysone, and GABA in the expression of the maternal effect. miR-125–5p was 1.8-fold more abundant in brains of nondiapause-history, short-day females than in the other three groups (Fig. 2). Only nondiapause-history, short-day females produce offspring with the potential to enter diapause, and we hypothesize that miR-125–5p is important for initiating diapause in *S. bullata*. We also predict that upstream regulation of miR-125–5p is an important part of the maternal effect. In *D. melanogaster* miR-125–5p is regulated by ecdysone, and in turn, targets the gene encoding Chronologically inappropriate morphogenesis (Chinmo) (Chawla and Sokol, 2012; Chawla et al., 2016; Pandey et al., 2021). Chinmo is a transcription factor that facilitates communication between the brain and fat body, and it negatively regulates transcription of genes involved in lipid metabolism (e.g., fatty acid transport protein, fatty acid synthase, acetyl-CoA oxidase, etc.). Several genes that are regulated by Chinmo (e.g., *fatty acid synthase 1*, fatty acid transport protein, acyl-CoA oxidase, enoyl-CoA hydratase, and others) are associated with lipid metabolism during diapause in beetles, silkworms, and mosquitoes, (Chen et al. 2017; Sim and Denlinger, 2009; Tan et al., 2017). Chinmo and its targets have not been formally studied in *S. bullata*, but it is likely they are important for regulating diapause in these flies as well.

Do changes in the circadian clock regulate the maternal effect?

We also measured transcript abundances of *period, timeless, clock, clockwork orange, vrille, and PAR domain protein* (*pdp*) in brains dissected from adult females to test the hypothesis that the *S. bullata* maternal effect is associated with changes in circadian clock function. Accumulating evidence from mosquitoes, flies, wasps, beetles, and moths suggests that circadian clock, and the genes that encode clock components (e.g., *period, timeless, cryptochrome 2*, and others) regulate diapause (Chang and Meuti, 2020; Dalla et al., 2019; Goto et al., 2006; Homma et al., 2022; Meuti, et al., 2015; Ren et al., 2018; Saunders, 2020; Zhu et al., 2019). Period and Timeless regulate lipid accumulation and metabolism in *Culex pipiens* mosquitoes and *Colaphellus bowringi* beetles (Meuti et al., 2015; Zhu et al., 2019). Clock, Clockwork orange, Vrille, and Par domain protein (PDP) have been implicated in seasonal responses in butterfly *Pararge aegeria*, but the nature of their involvement is not well defined (Lindestad et al., 2022).

We found significant differences in mRNA expression of *clock, period, timeless, clockwork orange,* and *vrille* in the brains of female *S. bullata* ($p \leq 0.05$; Fig. 3). Transcription of *timeless* and *vrille* was elevated in brains of short-day females compared to their long-day counterparts regardless of their diapause history, which suggests these genes are regulated more by photoperiod than diapause history. mRNA expression of *period, clock, and clockwork orange* appears to be regulated by an interaction between photoperiod and diapause history because differences observed between females that had experienced diapause (i.e., diapause-history, short-day and diapause-history, long-day females) were not observed in females with no diapause history (i.e. no diapause-history short-day and no diapause-history long-day females). *Period* abundance was 50 % lower in diapause-history, short-day females compared to their long-day counterparts. *Clock* and *Clockwork orange* abundances were elevated 2-fold in diapause-history, short-day females compared to diapause-history, long-day females. The biological significance of these differences is not clear, and more experiments are needed to solve this puzzle. We also need to consider how these genes are regulated post-transcriptionally. Accumulating evidence suggests that clock-related genes are regulated at

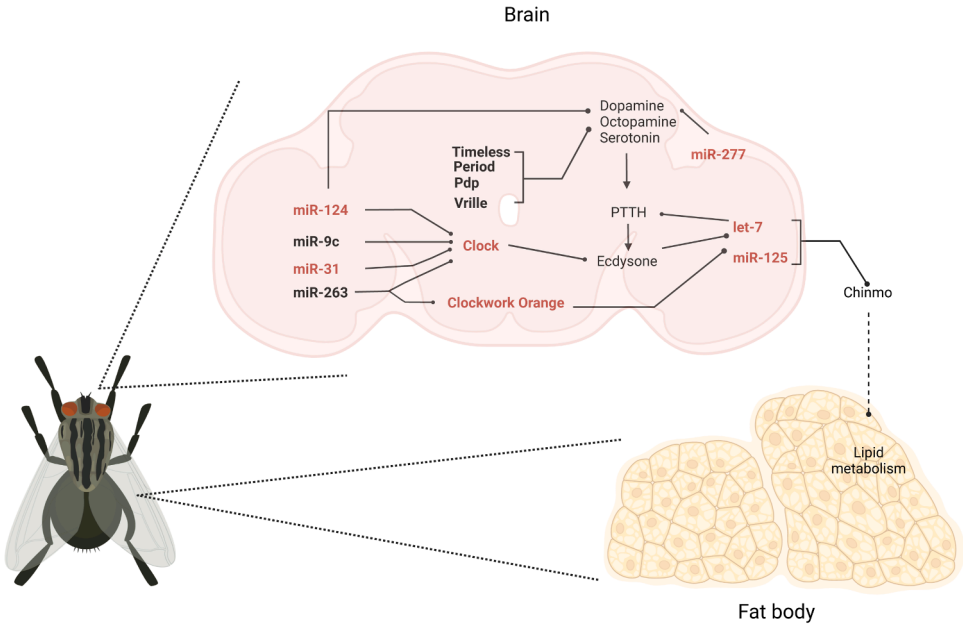

Fig. 4. Proposed network of microRNAs and genes that regulate diapause and/or the maternal block of diapause in *S. bullata*. Candidate genes and miRNAs evaluated in this study are indicated with bold. Differentially regulated genes and miRNAs are red, bolded text.

multiple levels including post-transcriptional regulation (Anna and Kannan 2021; Kadener et al., 2007, 2009; Parnell et al., 2021: Yang et al., 2008; You et al., 2018). Let-7–5p, miR-31–5p, and miR-124–3p all interact with clock-related genes (Fig. 4), and they are associated with changes in activity patterns and sleep-wake cycles. In addition, miR-9c-5p and miR-263–5p interact with Clock and Clockwork orange (Anna and Kannan, 2021; Kadener et al., 2009). The modest differences in miR-9c-5p and miR-263–5p were not statistically different (Supplementary Fig. S2), but we cannot rule out possible biological significance. These microRNAs and their associated circadian clock genes are also part of one, or more, regulatory networks that also include neurotransmitters and ecdysone (He et al., 2017). Future research will test hypotheses about the nature of these networks and how they regulate diapause and the maternal effect in *S. bullata*.

Conclusions

Differential regulation of microRNAs miR-277–3p, miR-125–5p, miR-31–5p, and miR-124–3p in the dissected brains of adult females that experienced diapause suggests they have a role regulating expression of the maternal block of diapause in *S. bullata*. In other insects these microRNAs are part of regulatory networks that also include circadian clock genes, neurotransmitters (e.g., GABA and Dopamine), and ecdysone. We predict that similar networks with one or more of these factors regulate diapause and the maternal effect in *S. bullata*. However, the moderate differences we found for *clock, clockwork orange*, and *period* are difficult to interpret, and we have not yet evaluated neurotransmitter synthesis or metabolism in this study. Our future work will investigate possible interactions between microRNAs (miR-125–5p, miR-277–3p, miR-31–5p, miR-124–3p, and miR-263–5p); circadian clock genes (*clock, period*, and *clockwork orange*), and downstream signaling by ecdysone, GABA, and dopamine.

Data availability

All data is available in the main text or in supplementary files. A file with raw processed data is provided.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Megan Meuti for sharing her knowledge about dissecting insect brains. Dr. Dave Denlinger provided valuable feedback on the manuscript. Alison Reynolds created the *S. bullata* graphics used in Fig. 1. Funding for this project was provided by the National Science Foundation (NSF_IOS 1755318 to J.A.R).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100099.

References

Anna, G., Kannan, N.N., 2021. Post-transcriptional modulators and mediators of the circadian clock. Chronobiol. Int. 38 (9), 1244–1261. https://doi.org/10.1080/07420528.2021.1928159.

Andreatta, G., Kyriacou, C.P., Flatt, T., et al., 2018. Aminergic signaling controls ovarian dormancy in drosophila. Sci. Rep. 8, 2030.

Beckwith, E.J., Gorostiza, E.A., Berni, J., Rezával, C., Pérez-Santángelo, A., Nadra, A.D., Ceriani, M.F., 2013. Circadian period integrates network information through activation of the BMP signaling pathway. PLoS. Biol. 11 (12), e1001733.

Chang, V., Meuti, M.E., 2020. Circadian transcription factors differentially regulate features of the adult overwintering diapause in the Northern house mosquito, Culex pipiens. Insect Biochem. Mol. Biol. 121, 103365. https://doi.org/10.1016/j.ibmb.2020.103365.

Chawla, G., Sokol, N.S., 2012. Hormonal activation of let-7-C microRNAs via EcR is required for adult Drosophila melanogaster morphology and function. Development 139, 1788–1797. https://doi.org/10.1242/dev.077743.

Chawla, G., Luhur, A., Sokol, N., 2016. Analysis of MicroRNA function in drosophila. Methods Mol. Biol. 1478, 79–94. https://doi.org/10.1007/978-1-4939-6371-3_4.

Chen, W., Liu, Z., Li, T., Zhang, R., Xue, Y., Zhong, Y., Bai, W., Zhou, D., Zhao, Z., 2014. Regulation of Drosophila circadian rhythms by miRNA let-7 is mediated by a regulatory cycle. Nat. Commun. 5, 5549. https://doi.org/10.1038/ncomms6549.

Chen, Y.R., Jiang, T., Zhu, J., Xie, Y.C., Tan, Z.C., Chen, Y.H., Tang, S.M., Hao, B.F., Wang, S.P., Huang, J.S., Shen, X.J., 2017. Transcriptome sequencing reveals potential mechanisms of diapause preparation in bivoltine silkworm Bombyx mori

(Lepidoptera: bombycidae). Comp. Biochem. Physiol. Part D. Genomics. Proteomics. 24, 68–78. https://doi.org/10.1016/j.cbd.2017.07.003.
Denlinger, D.L., 2022. Diapause among the flesh flies (Diptera: sarcophagidae). Eur. J. Entomol. 19, 170–182.
Denlinger, D.L., 1972. Induction and termination of pupal diapause in Sarcophaga (Diptera: sarcophagidae). Biol. Bull. 142, 11–24.
Denlinger, D.L., Campbell, J.J., Bradfield, J.Y., 1980. Stimulatory effect of organic solvents on initiating development in diapausing pupae of the flesh fly Sarcophaga crassipalpis and the tobacco hornworm Manduca sexta. Physiol. Entomol. 5, 7–15.
Foo, L.C., 2017. Cyclin-dependent kinase 9 is required for the survival of adult Drosophila melanogaster glia. Sci. Rep. 7, 6796. https://doi.org/10.1038/s41598-017-07179-8.
Fujiwara, Y., Denlinger, D.L., 2007. High temperature and hexane break pupal diapause in the flesh fly, Sarcophaga crassipalpis, by activating ERK/MAPK. J. Insect Physiol. 12, 1276–1282. https://doi.org/10.1016/j.jinsphys.2007.07.001.
Goto, S.G., Han, B., Denlinger, D.L., 2006. A nondiapausing variant of the flesh fly, Sarcophaga bullata, that shows arrhythmic adult eclosion and elevated expression of two circadian clock genes, period and timeless. J. Insect Physiol. 52, 1213–1218.
He, K., Sun, Y., Xiao, H., Ge, C., Li, F., Han, Z., 2017. Multiple miRNAs jointly regulate the biosynthesis of ecdysteroid in the holometabolous insects, Chilo suppressalis. RNa 23, 1817–1833. https://doi.org/10.1261/rna.061408.117.
Henrich, V.C., Denlinger, D.L., 1982. A maternal effect that eliminates pupal diapause in progeny of the flesh fly Sarcophaga bullata. J. Insect Physiol. 28, 881–884.
Homma, S., Murata, A., Ikegami, M., Kobayashi, M., Yamazaki, M., Ikeda, K., Daimon, T., Numata, H., Mizoguchi, A., Shiomi, K., 2022. Circadian clock genes regulate temperature-dependent diapause induction in silkworm Bombyx mori. Front. Physiol. 13, 863380. https://doi.org/10.3389/fphys.2022.863380.
Jin, X., Wu, X., Zhou, L., He, T., Yin, Q., Liu, S., 2020. 20-Hydroxyecdysone-responsive microRNAs of insects. RNA Biol. 17 (10), 1454–1471. https://doi.org/10.1080/15476286.2020.1775395.
Kadener, S., Menet, J.S., Sugino, K., Horwich, M.D., Weissbein, U., Nawathean, P., Vagin, V.V., Zamore, P.D., Nelson, S.B., Rosbash, M., 2009. A role for microRNAs in the Drosophila circadian clock. Genes Dev. 23, 2179–2191. https://doi.org/10.1101/gad.1819509.
Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M., 2007. Clockwork orange is a transcriptional repressor and a new Drosophila circadian pacemaker component. Genes Development 21, 1675–1686.
Kotwica-Rolinska, Joanna, Damulewicz, Milena, Chodakova, Lenka, Kristofova, Lucie, Dolezel, David, 2022. Pigment Dispersing Factor Is a circadian clock output and regulates photoperiodic response in the linden bug, Pyrrhocoris apterus. Front. Physiol. 13. https://doi.org/10.3389/fphys.2022.884909.
Kronholm, I., 2022. Evolution of anticipatory effects mediated by epigenetic changes. Environ. Epigenetics 8, 1–12. https://doi.org/10.1093/eep/dvac007.
Lee, I.H., Duvall, L.B, 2022. Maternally instigated diapause in Aedes albopictus: coordinating experience and internal state for survival in variable environments. Front. Behav. Neurosci. 16. https://doi.org/10.3389/fnbeh.2022.778264.
Li, Y., Haynes, P., Zhang, S.L., Yue, Z., Sehgal, A., 2023. Ecdysone acts through cortex glia to regulate sleep in Drosophila. Elife 12, e81723.
Lindestad, O., Nylin, S., Wheat, C.W., Gotthard, K., 2022. Local adaptation of life cycles in a butterfly is associated with variation in several circadian clock genes. Mol. Ecol. 31, 1461–1475. https://doi.org/10.1111/mec.16331.
Marshall, D.J., Uller, T, 2007. When is a maternal effect adaptive? Oikos. 116, 1957–1963. https://doi.org/10.1111/j.2007.0030-1299.16203.x.
Martinson, E.O., Peyton, J., Kelkar, Y.D., Jennings, E.C., Benoit, J.B., Werren, J.H., Denlinger, D.L., 2019. Genome and ontogenetic-based transcriptomic analyses of the flesh fly. Sarcophaga bullata. G3 (Bethesda) 9 (5), 1313–1320. https://doi.org/10.1534/g3.119.400148.
Meuti, M.E., Bautista-Jimenez, R., Reynolds, J.A., 2018. Evidence that microRNAs are part of the molecular toolkit regulating adult reproductive diapause in the mosquito, Culex pipiens. PLoS. One 13, e0203015. https://doi.org/10.1371/journal.pone.0203015.
Meuti, M.E., Stone, M., Ikeno, T., Denlinger, D.L., 2015. Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens. J. Exp. Biol. 218, 412–422. https://doi.org/10.1242/jeb.113233.
Mousseau, T.A., Fox, C.W., 1998. The adaptive significance of maternal effects. Trends. Ecol. Evol. 13, 403–407. https://doi.org/10.1016/s0169-5347(98)01472-4. PMID: 21238360.
Mukai, A., Mano, G., Des Marteaux, L., Shinada, T., Goto, S.G., 2022. Juvenile hormone as a causal factor for maternal regulation of diapause in a wasp. Insect Biochem. Mol. Biol. 144, 103758. https://doi.org/10.1016/j.ibmb.2022.103758.
Parnell, A.A., De Nobrega, A.K., Lyons, L.C., 2021. Translating around the clock: multi-level regulation of post-transcriptional processes by the circadian clock. Cell Signal. 80, 109904. https://doi.org/10.1016/j.cellsig.2020.109904.
Reznik, S.Y., Samartsev, K.G., 2015. Multigenerational maternal inhibition of prepupal diapause in two Trichogramma species (Hymenoptera: trichogrammatidae). J. Insect Physiol. 81, 14–20. https://doi.org/10.1016/j.jinsphys.2015.06.012.
Reynolds, J.A., 2024. MicroRNAs in the developmental toolbox - a comparative approach to understanding their role in regulating insect development. Curr. Opin. Insect Sci. https://doi.org/10.1016/j.cois.2024.101256. In press.
Reynolds, J.A., Clark, J., Diakoff, S.J., Denlinger, D.L., 2013. Transcriptional evidence for small RNA regulation of pupal diapause in the flesh fly, Sarcophaga bullata. Insect Biochem. Mol. Biol. 43, 982. https://doi.org/10.1016/j.ibmb.2013.07.005. -9.
Reynolds, J.A., Bautista-Jimenez, R., Denlinger, D.L., 2016. Changes in histone acetylation as potential mediators of pupal diapause in the flesh fly, Sarcophaga bullata. Insect Biochem. Mol. Biol. 76, 29–37. https://doi.org/10.1016/j.ibmb.2016.06.012.
Reynolds, J.A., Peyton, J.T., Denlinger, D.L., 2017. Changes in microRNA abundance may regulate diapause in the flesh fly, Sarcophaga bullata. Insect Biochem. Mol. Biol. 84, 1–14. https://doi.org/10.1016/j.ibmb.2017.03.002.
Rockey, S.J., Miller, B.B., Denlinger, D.L., 1989. A diapause maternal effect in the flesh fly, Sarcophaga bullata: transfer of information from mother to progeny. J. Insect Physiol. 35, 553–558.
Shen, Z.J., Liu, Y.J., Zhu, F., Cai, L.M., Liu, X.M., Tian, Z.Q., Cheng, J., Li, Z., Liu, X.X., 2020. MicroRNA-277 regulates dopa decarboxylase to control larval-pupal and pupal-adult metamorphosis of Helicoverpa armigera. Insect Biochem. Mol. Biol. 122, 103391. https://doi.org/10.1016/j.ibmb.2020.103391.
Sim, C., Denlinger, D.L., 2009. Transcription profiling and regulation of fat metabolism genes in diapausing adults of the mosquito Culex pipiens. Physiol. Genomics. 39, 202–209. https://doi.org/10.1152/physiolgenomics.00095.2009.
Tan, Q.Q., Liu, W., Zhu, F., Lei, C.L., Hahn, D.A., Wang, X.P., 2017. Describing the diapause-preparatory proteome of the beetle colaphellus bowringi and identifying candidates affecting lipid accumulation using isobaric tags for mass spectrometry-based proteome quantification (iTRAQ). Front. Physiol. 8, 251. https://doi.org/10.3389/fphys.2017.00251.
Tougeron, K., Hraoui, G., Le Lann, C., van Baaren, J., Brodeur, J, 2018. Intraspecific maternal competition induces summer diapause in insect parasitoids, 2018 Insect Sci. 25, 1080–1088. https://doi.org/10.1111/1744-7917.12491.
Wang, C., Feng, T., Wan, Q., Kong, Y., Yuan, L., 2014. miR-124 controls Drosophila behavior and is required for neural development. Int. J. Dev. Neurosci. 38, 105–112.
Yang, M., Lee, J.E., Padgett, R.W., Edery, I., 2008. Circadian regulation of a limited set of conserved microRNAs in drosophila. BMC Genomics 9, 83. https://doi.org/10.1186/1471-2164-9-83.
You, S., Fulga, T.A., Van Vactor, D., Jackson, F.R., 2018. Regulation of circadian behavior by astroglial microRNAs in drosophila. Genetics 208 (3), 1195–1207. https://doi.org/10.1534/genetics.117.300342.
Yu, Y., Zhang, X., Liu, F., et al., 2021. A stress-induced miR-31–CLOCK–ERK pathway is a key driver and therapeutic target for skin aging. Nat. Aging 1, 795–809.
Zhang, Y., Lamba, P., Guo, P., Emery, P., 2016. miR-124 Regulates the phase of Drosophila circadian locomotor behavior. J. Neurosci. 36 (6), 2007–2013. https://doi.org/10.1523/JNEUROSCI.3286-15.2016.