Entrainment of eclosion and preliminary ontogeny of circadian
clock gene expression in the flesh fly, *Sarcophaga crassipalpis*

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Abstract

Timing of circadian activities is controlled by rhythmic expression of clock genes in pacemaker neurons in the insect brain. Circadian behavior and clock gene expression can entrain to both thermoperiod and photoperiod but the availability of such cues, the organization of the brain, and the need for circadian behavior change dramatically during the course of insect metamorphosis. We asked whether photoperiod or thermoperiod entrains the clock during pupal and pharate adult stages by exposing flies to different combinations of thermoperiod and photoperiod and observing the effect on the timing of adult eclosion. This study used qRT-PCR to examine how entrainment and expression of circadian clock genes change during the course of development in the flesh fly, *Sarcophaga crassipalpis*. Thermoperiod entrains expression of *period* and controls the timing of adult eclosion, suggesting that the clock gene *period* may be upstream of the eclosion pathway. Rhythmic clock gene expression is evident in larvae, appears to cease during the early pharate adult stage, and resumes again by the time of adult eclosion. Our results indicate that both patterns of clock gene expression and the cues to which the clock entrains are dynamic and respond to different environmental signals at different developmental stages in *S. crassipalpis*.

Keywords: eclosion rhythm, rhythmicity, photoperiod, thermoperiod, *timeless*, *period*

1. Introduction

Plants and animals adjust their behavior and physiology to anticipate both favorable conditions and stresses such as temperature, desiccation, and predation that correspond to diel cycles (Tanaka & Watari, 2009; Parecis-Silva et al., 2016). The timing of these preparatory changes in physiology and behavior is programmed by the circadian clock. Molecular components of the insect circadian clock found across taxa include the genes *period*, *timeless*, *clock*, *cycle*, *cryptochrome 1*, and *cryptochrome 2*, although *cryptochrome 2* is absent in *Drosophila melanogaster* and other higher Diptera (Goto, 2013), while *timeless* and *cryptochrome 1* are absent in Hymenoptera (Zhan et al., 2011). These genes form a negative transcriptional feedback loop, resulting in *per* and *tim* mRNA levels that oscillate with peak expression shortly after lights-off (Yuan et al., 2007; Gentile et al., 2009; Goto & Denlinger, 2002). PER and TIM proteins form a heterodimer that translocates to the nucleus (Rutila et al., 1996; Shafer et al., 2002), but these two proteins can also act independently. PER can enter the nucleus before TIM and repress transcription *in vitro* and *in vivo* in the absence of TIM, although the two proteins do enhance each other's stability (Ashmore et al., 2003; Rothenfluh et al., 2000; Weber & Kay 2003). In *D. melanogaster* deprived of light cues, temperature entrainment of the clock leads to decoupling of *per* and *tim* expression by advancing *per* expression and delaying *tim* expression, though this divergence is absent during the free-running period following temperature entrainment (Boothroyd et al., 2007). The clock genes *clockwork orange*, *vriille*, and *Par domain protein 1ε* form additional loops of oscillatory expression in *D. melanogaster* (Tomioka & Matsumoto, 2015).

Although entrainment to photoperiod requires no input from peripheral tissues in *D. melanogaster*, entrainment to thermoperiod requires transmission of temperature signals from the chordotonal organs to the clock neurons within the brain (Sehadova et al., 2009). A temperature receptor in peripheral tissues may activate pacemaker neurons to entrain the molecular clock to temperature (Hamada et al., 2008), but much less is known about the conduits of thermoperiodic entrainment of the clock than is known about photoperiodic entrainment. The brain, a major site of clock gene expression (Kostal et al., 2009), undergoes extensive reorganization during metamorphosis (Fraenkel & Hsiao, 1968), and it is not clear how the reorganization of the brain impacts clock function during this transition.

The mechanism of entrainment, or how the circadian clock adjusts according to environmental signals, is only partially understood. The circadian clock entrains to photoperiod via the action of CRY1, which degrades TIM in the presence of blue light (Yuan et al., 2007). In addition to light, thermoperiod can also be a powerful *Zeitgeber*. Very small oscillations in temperature can entrain the circadian clock, including activity entrainment by oscillations of only 2°C in *D. melanogaster* (Wheeler et al., 1993), and eclosion entrainment by oscillations of only 0.4°C in the tsetse fly *Glossina morsitans* (Žďárek & Denlinger, 1995). In *D. melanogaster*, although many clock gene transcripts oscillate in response to thermoperiod (Boothroyd et al., 2007), the mechanisms entraining circadian clock gene expression to temperature are poorly understood. The clock gene *Clock* (*dClk*) is necessary to entrain other clock genes to thermoperiod in *D. melanogaster*, though *per* seems to be expressed independently of *dClk* (Yoshii et al., 2007). In *D. melanogaster* thermoperiod is able to entrain the expression of *per*, suggesting an important role for *per* in thermoperiod sensitivity (Glaser & Stanewsky, 2005). Low temperatures promote 3' intronic splicing of *per* in *D. melanogaster* (Majercak et al., 2004),

leading to faster *per* mRNA accumulation. The two isoforms of *timeless* also display changes in relative abundance under low temperatures, though the effect of alternative splicing of *timeless* is unknown (Boothroyd et al., 2007).

Adult eclosion is a distinct and well-studied circadian event in the life history of flies and most other insects. The gating of eclosion is controlled by a circadian clock that is sensitive to both thermoperiod and photoperiod (Myers, 2003). However, *tim*-null mutants of the fruit fly *D. melanogaster* still display rhythmic eclosion (Wuelbeck et al., 2005). This suggests that other genes in the clock regulate this response or perhaps there is some redundancy in the genes regulating the eclosion timing pathway. The specific clock genes that regulate eclosion and the mechanisms underlying clock function in the absence of TIM are not known. It is likely that regulation and interaction of circadian clock genes change over time depending on both the insect's phase of development and prevailing environmental signals. Many Diptera burrow underground during the wandering larval stage to pupariate. They thus undergo metamorphosis beneath the substrate, where photoperiodic signals are dampened or eliminated and temperature fluctuations are reduced. During metamorphosis the flesh fly, *Sarcophaga crassipalpis*, like most flies, is immobile and lacks overt behavior, suggesting that there may be no need for circadian rhythmicity until the time of eclosion.

The entrainment of eclosion by thermoperiod and photoperiod varies among insects. In the cricket *Gryllus bimaculatus*, removal of the optic lobes causes a switch in entrainment from light signals to temperature cues (Karpova & Tomioka, 2006). The drosophilid fly *Chymomyza costata* has more synchronous eclosion in a thermoperiodic regime, but weakly rhythmic eclosion is still determined by photoperiod in the absence of thermoperiodic cues (Lankinen & Riihimaa, 1997). The onion fly, *Delia antiqua* has an eclosion rhythm that is entrained by both

temperature and light cycles interacting in a manner consistent with the two oscillator model, where the cue with the greater amplitude overrides the weaker cue (Watari & Tanaka, 2010; Pittendrigh & Bruce, 1959). However, in *Glossina morsitans* thermoperiod overrides photoperiod to entrain eclosion, suggesting a single oscillator model where only one cue is responsible for entrainment (Ždárek & Denlinger, 1995).

The life stage that is sensitive to cues entraining eclosion varies among insects. Manipulating the thermoperiod experienced during the pharate adult stage of *D. antiqua* alters the adult eclosion rhythm (Watari, 2005). In the alfalfa leafcutter bee *Megachilidae rotundata*, thermoperiod during metamorphosis entrains the time of eclosion (Yocum et al., 2016). Alternatively, the eclosion rhythm may be entrained before pupariation. Saunders (1976) exposed the flesh fly *S. argyrostoma* to a 7 hour delay in light cycle at different life stages and found photoperiod during larval development determines eclosion rhythm, but during pupal and pharate adult development photoperiod is unable to synchronize eclosion. The Australian sheep blowfly *Lucilia cuprina* is sensitive to photoperiodic cues entraining eclosion only during its larval stages, as is also suggested for the flesh fly *S. crassipalpis* (Smith, 1985; Joplin & Moore, 1999). However, the entrainment of eclosion in *S. crassipalpis* is a point of contention, as Miyazaki et al. (2011) reported that, following pupal diapause, temperature oscillations during metamorphosis control the time of adult eclosion in this species.

Little information is available on the role of clock gene expression around the time of adult eclosion, nor have many investigations focused on the effects of conflicting photoperiodic and thermoperiodic cues on adult eclosion. Our study addresses these questions in the flesh fly *S. crassipalpis*. We hypothesized that the expression of circadian clock genes changes throughout the life of *S. crassipalpis*, displaying limited rhythmicity from pupariation until the early pharate

adult stage. We used quantitative Real Time-PCR (qRT-PCR) to record changes in *per* and *tim* expression between lights-on and lights-off at different life stages to form a tentative ontogeny of clock gene expression in *S. crassipalpis*. We then placed groups of *S. crassipalpis* in agreeing and conflicting thermoperiodic and photoperiodic conditions to determine whether light or temperature is the dominant signal controlling the timing of adult eclosion. Finally we measured changes in *per* expression during entrainment of adult eclosion by thermoperiod and photoperiod. We suggest that thermoperiod is the dominant environmental cue regulating the timing of adult eclosion and that the pattern of *per* expression plays a prominent role in coordinating this event.

2. Materials and methods

2.1. Insects and rearing conditions

A colony of *S. crassipalpis* Macquart originating from adults collected in Worthington, Ohio in the summer of 2014 was maintained as described (Denlinger, 1972). For qRT-PCR experiments to determine clock ontogeny, flies larviposited on a piece of beef liver and larvae were reared under a 16:8 LD (light:dark) cycle at 22°C and then collected at 4 different developmental time points: (1) wandering third-instar larvae, (2) early pharate adults corresponding to 7 days after pupariation, and (3) late pharate adults (i.e. black bristle stage) corresponding to 1 day prior to eclosion. Remaining puparia were transferred to 11:13 LD at 18°C and a further group (4) consisted of adults collected 14 days after eclosion.

2.2. Determining entrainment of eclosion

To determine the relative contributions of thermoperiod and photoperiod to entrainment of adult eclosion flies were transferred at pupariation to one of five combinations of

thermoperiod and photoperiod detailed in Table 1. In brief: (1) photoperiod and thermoperiod in agreement (16:8 LD with 16:8 25°C thermophase:18°C cryophase [TC] cycle); (2) photoperiod and thermoperiod in conflict (16:8 LD with 16:8 18°C cryophase:25°C thermophase cycle); (3) photoperiod alone (16:8 LD at constant 22°C temperature); (4) thermoperiod alone (16:8 TC 25°C:18°C cycles in constant darkness [DD]); and (5) no temperature or light cues (DD at 22°C) (n=50 for all groups). Two days before adult eclosion flies were transferred from the above conditions to constant darkness and 20°C. In these conditions the puparia were placed in an experimental apparatus inside 0.2 mL test tubes with a small metal ball (air gun shot, 4.5mm) placed at the mouth of each test tube. Eclosing flies bumped the bead into a fraction collector (Isco model 1850) set to rotate every 32 minutes, as described (Saunders, 1978; Yocum et al., 1994). With this apparatus the rhythmicity of eclosion could be measured without disturbing the flies or exposing them to unnecessary temperature and light cues.

Table 1. The combinations of thermoperiod and photoperiod used for eclosion experiments. Bars on top indicate photoperiod, with yellow indicating lights-on and grey indicating lights-off. The bars below indicate thermoperiod, with red bars indicating thermophase, blue bars indicating cryophase, and green bars indicating a constant temperature of 22°C.

Trial condition	Combination of thermoperiod and photoperiod	
Photoperiod and thermoperiod in agreement	16 hours lights on	8 hours lights off
	16 hours at 25°C	8 hours at 18°C
Photoperiod and thermoperiod in conflict	16 hours lights on	8 hours lights off
	16 hours at 18°C	8 hours at 25°C
Photoperiod alone	16 hours lights on	8 hours lights off
	24 hours at 22°C	
Thermoperiod alone	24 hours lights off	
	16 hours at 25°C	8 hours at 18°C
Neither thermoperiod nor photoperiod	24 hours lights off	
	24 hours at 22°C	

2.3. RNA extraction, cDNA synthesis and qRT-PCR

To monitor ontogeny of clock gene cycling, wandering larvae, early pharate adults, and late pharate adults were collected at lights-on (*Zeitgeber* Time 0 [ZT]) and lights-off (ZT16). Flies (n=4, each a pooled sample consisting of 3 heads) collected during these stages were frozen at -80°C until decapitation and RNA extraction. During the larval and early adult stages the head contains a considerable amount of adipose tissue which could possibly obscure the qRT-PCR signal. The validity of using entire heads of larvae and early pharate adults frozen at -80°C was checked by also preserving these stages in RNAlater® and dissecting the brains. The posterior half of the larvae and early pharate adults were removed during sampling to allow penetration of RNAlater® into the tissue. Samples were stored for 1-3 weeks in 1mL of RNAlater® at 4°C, then the brains were dissected in 100% ethanol to collect the central brain and optic lobes (n=4 for all groups, each a pooled sample of 5 brains). RNA was extracted using TRIzol® (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript® III First-Strand Synthesis Reagents (Invitrogen) according to the manufacturer's instructions using 1 µg of total RNA per 20 µg reaction. *per*, *tim*, and *cry1* were measured using qRT-PCR according to Meuti et al. (2015) although results were normalized to *cry1* rather than *rp49*, as *cry1* is expressed constitutively in the brain of *S. crassipalpis* (Goto & Denlinger, 2002); and *D. melanogaster* (Veleri & Wulbeck, 2004). We also measured *cry1* expression in our samples and found that it was not significantly different between ZT0 and ZT16 at any developmental stage sampled (Student's t-test, df=5, p>0.19 for all developmental stages). Primers are listed in Table 2.

One day before adult eclosion flies were removed from the five rearing conditions described above at both ZT0 (onset of photophase or thermophase) and ZT16 (beginning of scotophase or cryophase), or in groups without thermoperiod or photoperiod, at the equivalent Circadian Times (CT0 and CT16), and frozen at -80°C (n=4, each a pooled sample of 3 heads). RNA extraction, cDNA synthesis and qRT-PCR were run in the same manner as the ontogeny experiments. We again confirmed that *cry1* expression was not significantly different between ZT0 and ZT16 under any rearing condition (Student's t-test, df=5, $p>0.25$ for all developmental time points), and hence was a reliable reference gene for qPCR normalization.

Table 2

Sequence of primers

Oligonucleotides	Sequence (5'→3')
<i>S. c. tim1</i> FWD	CTACCTCGGTTGACAGAACTAAC
<i>S. c. tim1</i> REV	CCAGTAGAGTGTGTCCATTTC
<i>S. c. per4</i> FWD	GCACGAATTGGTCGTATTAAAG
<i>S. c. per4</i> REV	GACACCTTCTAGAAACCTCTTG
<i>S. c. cry1.4</i> FWD	GGGAACATGGCTTACGATATT
<i>S. c. cry1.4</i> REV	AAATGCCGACGAACTAACC

2.4 Statistical tests

To test synchrony of eclosion the number of flies that eclosed between ZT20 and ZT4 (corresponding to 4 hours before and after either the onset of photophase or thermophase) was compared to the number of flies that eclosed outside this window (ZT4 until ZT20) using the Fisher exact test ($p<0.05$ indicates significantly synchronous eclosion). In cases where temperature and light cues conflicted, the windows corresponded to ZT12 until ZT20 (4 hours before and after the onset of thermophase) and ZT20 until ZT12. The number of flies within a

given window were also compared with the number outside this window using parameter R, a scalar statistic in which low values indicate synchronous eclosion and high values indicate asynchronous eclosion ($R = [N_{\text{in window}} / N_{\text{out of window}}] * 100$) (Winfree, 1970; Saunders, 1976; Smith, 1985).

qRT-PCR fluorescence data was collected in the BioRad program and a curve was generated by MATLAB (MathWorks®) according to the program described by Meuti et al. (2015) and normalized to *cry1* as described above. Relative biological expression of *per* and *tim* at lights-on (ZT0) and lights-off (ZT16) was compared using the Student's t-test (Microsoft Excel®). p values below 0.05 were considered significant.

3. Results

3.1. Dynamics of clock gene expression during fly metamorphosis

qRT-PCR of *period* and *timeless* was performed using flies raised under a 16:8 LD cycle at 22°C sampled at select developmental stages from the wandering larval stage until after adult eclosion at lights-on (ZT0) and lights-off (ZT16), as shown in Fig 1. Levels of *tim* expression were significantly higher at lights-off than lights-on in third-instar larvae (Relative fold change=9.0; Student's t-test, df=5, p=0.03), late pharate adults (Relative fold change=3.5; Student's t-test, df=5, p=0.02), and in adults (Relative fold change=2.9; Student's t-test, df=5, p<0.01). Similarly, expression of *per* was significantly higher at lights-off than at lights-on in late pharate adults (Relative fold change=1.3; Student's t-test, df=5, p=0.03) and in adults (Relative fold change=4.3; Student's t-test, df=5, p<0.01). However, the levels of *tim* and *per* expression did not change between lights-on and lights-off in early pharate adult brains or whole heads (Student's t-test, df=5, p=0.59; data for whole heads not shown). Additionally, *per*

expression did not change between lights-on and lights-off in wandering third-instar larvae (Student's t-test, $df=5$, $p=0.91$).

Fig. 1. Relative fold change in expression of *per* and *tim* between lights-on and lights-off at various life stages. Levels of *per* and *tim* mRNA were measured with qPCR and normalized to *cry1* expression. Fold change was calculated by dividing the expression of *per* and *tim* at lights-off (predicted maximum expression) by the expression of each gene at lights-on (predicted minimum expression). The p-values from the Student's t-test comparing relative expression of each gene between the lights-on and lights-off are shown above the bars. Error bars indicate standard deviation.

Effects of photoperiod and thermoperiod on adult eclosion and clock gene expression

Adult eclosion was measured in groups of flies reared under various combinations of photoperiodic and thermoperiodic conditions (Figs. 2 and 3). The number of flies that eclosed

between ZT20 and ZT4 (corresponding to 4 hours before until 4 hours after the onset of photophase or thermophase) was compared to the number of flies that eclosed outside this window (ZT4 until ZT20) using the Fisher exact test ($p < 0.05$ indicates significantly synchronous eclosion). Flies eclosed synchronously when they were exposed to agreeing thermoperiod and photoperiod ($R=2$; Fisher's exact test, $p < 0.01$) and thermoperiod alone ($R=12$; Fisher's exact test, $p=0.02$). When photoperiod and thermoperiod conflicted, flies eclosed synchronously in response to thermoperiod ($R=3$; Fisher's exact test, $p=0.01$). Both agreeing and conflicting conditions of photoperiod and thermoperiod resulted in peak eclosion occurring during the transition from cryophase to thermophase, irrespective of lighting regimes. There was no significant synchrony of eclosion when flies were exposed to photoperiodic signals alone ($R=41$; Fisher's exact test, $p > 0.05$) or in flies receiving neither thermoperiodic nor photoperiodic cues ($R=30$; Fisher's exact test, $p > 0.05$).

Fig. 2. Number of flies eclosing throughout the day under varying temperature and light regimes corresponding to only thermoperiod, only photoperiod, both cues, or neither cue. *Zeitgeber* Time indicates either lights-on or the onset of thermophase, while Circadian Time is the subjective start of these cues for flies raised in the absence of photoperiodic or thermoperiodic cues.

Colored bars beneath the graph correspond to the combination of cues in which flies were reared, with filled bars indicating photoperiod (open bars indicate photophase, filled bars indicate scotophase) and cross-hatching indicating thermoperiod (cross-hatching indicates 25°C, no cross-hatching indicates 18°C, stippling indicates 22°C).

Fig. 3. Number of flies that eclosed throughout the day under agreeing and conflicting thermoperiod and photoperiod. Bars beneath the graph indicate the combination of cues in which flies were reared, with shading indicating photoperiod, (open bars indicate photophase; filled

bars indicate scotophase) and cross-hatching indicating thermoperiod (cross-hatching indicates thermophase, no cross-hatching indicates cryophase).

In addition to measuring the effects of thermoperiod and photoperiod on adult eclosion, we also examined how these signals impacted circadian clock gene expression. One day before eclosion the level of *per* mRNA was measured using qRT-PCR normalized to *cry1* (Fig. 4). Transcription level of *per* at ZT0 (the beginning of photophase and/or thermophase) and ZT16 (the beginning of scotophase and/or cryophase) were compared. We found significantly higher *per* expression at ZT16 relative to ZT0 in flies exposed to agreeing thermoperiod and photoperiod (Relative fold change=1.3; Student's t-test, df=5, p=0.03) and only thermoperiod (Relative fold change=2.5; Student's t-test, df=5, p=0.04). However, when flies were reared under conflicting photoperiodic and thermoperiodic conditions, *per* expression reversed, with significantly higher expression levels at ZT0 (lights-on and the onset of cryophase) relative to ZT16 (lights-off and the onset of thermophase) (Relative fold change=1.2; Student's t-test, df=5, p=0.03). Therefore, when thermoperiodic and photoperiodic cues were in conflict, *per* expression responded to thermoperiod. *per* expression did not change between ZT0 and ZT16 when flies were exposed to photoperiod alone (Student's t-test, df=5, p=0.95) or between CT0 and CT16 when they were reared in the absence of both thermoperiodic and photoperiodic cues (Student's t-test, df=5, p=0.54).

Fig. 4. Relative fold change in expression of *per* at two times of the day in flies reared under various combinations of thermoperiod and photoperiod. Levels of *per* mRNA were measured with qPCR and normalized to *cry1* expression. Fold change was calculated by dividing expression of *per* at ZT16 (the beginning of scotophase and/or cryophase; predicted maximum expression) by its expression at ZT0 (the beginning of the photophase and/or thermophase; predicted minimum expression) for flies reared with photoperiod alone, thermoperiod alone and agreeing thermoperiodic and photoperiodic regimes. Expression of *per* at the same relative time points (CT16 and CT0) was monitored in conditions when the flies were reared in the absence of photoperiodic or thermoperiodic cues. When photoperiod and thermoperiod conflicted, the relative expression of *per* at ZT0 (lights-on and onset of cryophase; observed maximum expression) was divided by expression at ZT16 (Lights-off and the onset of thermophase; observed minimum expression). The p-values from the Student's t-test comparing the relative

expression of *per* between the time of ZT0 (or CT0) and ZT16 (or CT16) are shown above the bars. Error bars indicate standard deviation.

4. Discussion

Flesh flies, like most insects, have differing needs for circadian behavior throughout development, and the environments to which they are exposed at different times offer diverse heat and light cues. This study sampled flies at several developmental stages at both lights-on (ZT0) and lights-off (ZT16) to form a tentative ontogeny for circadian clock gene cycling. The developmental stages sampled do not represent all life stages of *S. crassipalpis*, and sampling at only lights-on and lights-off cannot distinguish lack of rhythmicity from phase shifts. Therefore the ontogeny of clock gene cycling presented serves as a preliminary framework suggesting that the clock is dynamic during metamorphosis in *S. crassipalpis*. Kostal et al. (2009) reported rhythmic *per* and *tim* mRNA expression in first-instar larvae of *S. crassipalpis*. We found rhythmicity in *tim* but not *per* expression in third-instar larvae of *S. crassipalpis*, supporting the hypothesis that *per* and *tim* function independently at certain developmental stages. Our results suggest that rhythmicity ceased early in the pharate adult stage, although it is also possible that circadian clock gene expression shifted during this point in development, and our limited sampling precluded us from finding altered rhythmic expression. However this apparent loss in rhythmic expression could also be associated with desynchronization of the pacemaker neurons (Hong & Saunders, 1998). The apparent loss of clock gene cycling around the time of pupariation may represent a transitional phase as the interaction between PER and TIM changes or as neurons expressing clock genes desynchronize. Brain morphology changes dramatically in flesh flies during metamorphosis (Fraenkel & Hsiao, 1968), and the onset of metamorphosis is

thus a likely time for rearrangement of neurons and their subsequent desynchronization.

Desynchronization may be associated with changes in which sets of neurons display a functional circadian clock. Circadian neurons in the brain of *Drosophila* are well-mapped and characterized, and the circadian neurons fall into two groups: those active in the first-instar, called early pacemaker neurons, and those that remain inactive until late pupal stages, the late pacemaker neurons (Kaneko et al., 1997). This latter group comprises 85% of the neurons within the brain (Kaneko & Hall, 2000). The late pacemaker neurons present in third-instar *D. melanogaster* larvae express CLK and CYC, but are unable at this stage to generate rhythmic *per* and *tim* expression, suggesting that some cue experienced in the late pupal or pharate adult stage is required for CLK-CYC and PER-TIM to interact (Liu et al., 2015). We observed rhythmic expression of *per* and *tim* at lights-on and lights-off in late pharate adults of *S. crassipalpis*. Clock gene cycling thus resumed normal expression prior to the day before eclosion, suggesting that the *D. melanogaster* model of pacemaker neuron development may also apply to *S. crassipalpis*. As expected, adults of *S. crassipalpis* exhibited rhythmic expression of both *per* and *tim*. The rhythmic expression of clock genes in adults just prior to eclosion and after eclosion is not surprising, as circadian genes regulate the gated event of synchronous eclosion as well as rhythmic behaviors including feeding, resting, and mating (Mistlberger, 1994; Smith & Konopka, 1982; Seghal et al., 1994).

Our finding that the circadian clock may stop during early pharate adult development in *S. crassipalpis* implies that the clock is unlikely to cycle during the overwintering period of pupal diapause in this species. Periods of dormancy may or may not involve a stop in clock function. Although circadian oscillations cease in hibernating hamsters (Revel et al., 2007), diapausing adults of the mosquito *Culex pipiens* continue to show oscillations of clock gene

expression during diapause (Meuti et al., 2015). These distinctions presumably depend on the behavior and stage of the dormant animal. While flesh fly pupae and hibernating hamsters have severely restricted behavioral repertoires during dormancy, the diapausing mosquito adults remain mobile and continue to seek locations with high humidity throughout the winter months and rely on photoperiodic cues for diapause termination in the spring.

Prior to adult eclosion, rhythmic clock gene expression resumes in response to the same cues that entrain the eclosion rhythm. Our findings indicate that thermoperiod governs adult eclosion and is a more powerful *Zeitgeber* than light because photoperiod alone did not entrain synchronous eclosion. Parameter R values, scalar statistics in which low values indicate synchronous eclosion, demonstrate that although many flies emerged early in the photophase when exposed to photophase alone the R value was quite high (R=41). A much lower R value was seen in groups of flies reared in the absence of thermoperiod and photoperiod (R=30), confirming that light is not an effective *Zeitgeber* during metamorphosis (Winfree, 1970; Saunders, 1976; Smith, 1985). Our findings contradict the idea that the eclosion rhythm in *S. crassipalpis* follows a two oscillator model described by Pittendrigh and Bruce (1959), in which the dominant *Zeitgeber* is determined by signal strength. Thermoperiod alone was shown to entrain adult eclosion in *S. crassipalpis* (R=12), as was thermoperiod in conflict with photoperiodic cues (R=3). This suggests that the eclosion rhythm in *S. crassipalpis* is driven by a single oscillator and thermoperiod is the only cue responsible for entrainment.

Controversy over which life stage of *S. crassipalpis* is sensitive to entrainment may stem from the statistical tests used to detect significant eclosion synchrony. Parameter R suggests flies that experience photoperiod during larval development display synchronous eclosion regardless of photoperiodic and thermoperiodic conditions experienced during metamorphosis, consistent

with the results of Joplin & Moore (1999) who found photoperiod during the third-instar larval stage of *S. crassipalpis* larvae entrains rhythmic adult eclosion. Our results also showed peaks of eclosion, though broad, for groups deprived of thermoperiodic cues. These peaks of eclosion may have been entrained by photoperiod in the third instar as predicted by Joplin & Moore (1999). However, when a stricter statistical analysis, Fisher's exact test, is used, only groups exposed to thermoperiod during metamorphosis display synchronous eclosion. Given that parameter R values were the lowest in groups that were exposed to thermoperiod during metamorphosis, flies are clearly responsive to cues experienced outside the three-day photosensitive window at the end of the larval stage that was reported by Joplin & Moore (1999). It is also possible that the rhythmic eclosion pattern detected by Joplin & Moore (1999) was due to inadvertent fluctuations in temperature during metamorphosis rather than the photoperiodic conditions flies experienced as third-instar larvae; some other dipterans, such as the tsetse fly, can entrain to temperature oscillations as small as 0.4°C (Žďárek & Denlinger, 1995), a temperature difference small enough to be feasibly induced by minor temperature changes in the rearing room. Our findings that *S. crassipalpis* can entrain to thermoperiodic signals in late pharate adult development is consistent with the findings of Miyazaki et al. (2011).

A correctly functioning circadian clock is critical for survival of *S. crassipalpis*, but from the time of pupariation until the time of adult eclosion the fly is immobile and lacks overt behavior. The lack of circadian behavior is reflected in the apparent lack of rhythmic clock gene expression. However, by the time of eclosion selective pressure for circadian behavior may have resumed, as a properly timed adult eclosion is conducive to proper wing expansion and preventing desiccation (Pittendrigh, 1954, Tanaka & Watari, 2009). Synchronized eclosion can also be expected to result in mass emergence of adult flies that cannot possibly all be eaten by a

predator, and synchrony is likely to also facilitate mating (Gillott, 1995). Thus, in our preliminary model, the clock appears to stop cycling during much of metamorphosis, but restarts in time to monitor thermoperiod and thereby synchronize adult eclosion.

The interaction between thermoperiod and photoperiod to determine eclosion rhythm is reflected in the expression pattern of the clock gene *period*. When exposed to conflicting thermoperiod and photoperiod, expression of *per*, but not *tim* (data not shown), was entrained by thermoperiod in *S. crassipalpis*. These results are consistent with observations in *D. melanogaster*, in which *per* has a similar expression pattern and oscillates independently of *tim* and in response to thermoperiod (Yoshii et al., 2007). *Drosophila melanogaster cry1* and *tim* mutants still exhibit rhythmic eclosion (Dolezelova et al., 2007; Wuelbeck et al., 2005). CRY1 degrades TIM when exposed to light to entrain the circadian clock to photoperiodic cues, thus if only thermoperiod regulates the eclosion rhythm, it is not surprising that *cry1* and *tim* mutants retain rhythmic eclosion. However, mRNA levels promote early eclosion in *D. melanogaster* while eliminating the *per* locus results in arrhythmic eclosion (Smith & Konopka, 1981; 1982). Given that both *per* expression and the eclosion rhythm are regulated by thermoperiod and not photoperiod, *per* is a likely upstream regulator of the eclosion pathway in *D. melanogaster* and *S. crassipalpis*, and possibly other dipterans as well.

The ontogeny we propose is based on a limited number of developmental and circadian time points and should be considered preliminary. A full circadian ontogeny based on a complete selection of developmental points and circadian time points would advance chronobiology and developmental biology. Our results indicate that thermoperiod during metamorphosis can override photoperiod to determine eclosion rhythm. Comparing the effect of both temperature and light cues on multiple circadian behaviors throughout an animal's life may reveal how the

entrainment of circadian behaviors changes during ontogeny and how underlying clock machinery allows an animal to adapt its behavior to rhythmic environments. Our results from this study suggest the merit of such future research by proposing a tentative model of dynamic clock gene ontogeny that can entrain behavior to temperature cues in addition to light cues.

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References

- Ashmore, L. J., Sathyanarayanan, S., Silvestre, D.W., Emerson, M.M., Schotland, P., & Sehgal, A. (2003). Novel insights into the regulation of the timeless protein. *Journal of Neuroscience*, 23, 7810-7819. doi: 10.3410/f.1014134.196012.
- Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., & Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *Plos Genetics*, 3, e54. doi:10.1371/journal.pgen.0030054
- Denlinger, D.L. (1972). Induction and termination of pupal diapause in *Sarcophaga* flesh flies. *Biological Bulletin*, 142, 11-24.
- Dolezelova, E., Dolezel, D., & Hall, J. C. (2007). Rhythm defects caused by newly engineered null mutations in *Drosophila*'s cryptochrome gene. *Genetics*, 177, 329-345. doi:10.1534/genetics.107.076513.
- Fraenkel, G., & Hsiao, C. (1968). Morphological and endocrinological aspects of pupal diapause in a fleshfly, *Sarcophaga agryrostoma*. *Journal of Insect Physiology*, 14, 707-718. doi: 10.1016/0022-1910(68)90229-1.
- Gentile, C., Rivas, G. B. S., Meireles-Filho, A. C. A., Lima, J. B. P., & Peixoto, A. A. (2009). Circadian expression of clock genes in two mosquito disease vectors: *Cry2* is different. *Journal of Biological Rhythms*, 24, 444-451. doi:10.1177/0748730409349169
- Gillott, C. (1995). *Entomology*. New York: Plenum Press.

- Glaser, F.T., & Stanewsky, R. (2005). Temperature synchronization of the *Drosophila* circadian clock. *Current Biology*, 15, 1352-1363. doi:10.1016/j.cub.2005.06.056
- Goto, S.G., & Denlinger, D.L. (2002). Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. *Journal of Insect Physiology*, 48, 803-816. doi:10.1016/S0022-1910(02)00108-7
- Goto, S. G. (2013). Roles of circadian clock genes in insect photoperiodism. *Entomological Science*, 16, 1-16. doi:10.1111/ens.12000
- Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J., & Garritty, P. A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature*, 454, 217-U55. doi:10.1038/nature07001
- Hong, S.F., & Saunders, D.S. (1998). Internal desynchronisation in blow fly (*Calliphora vicina*) locomotor activity rhythms: Evidence for a complex circadian pacemaker. *Biological Rhythm Research*, 29, 387-396. doi:10.1076/brhm.29.4.387.1432
- Joplin, K.H., & Moore, D. (1999). Effects of environmental factors on circadian activity in the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology*, 24, 64-71. doi:10.1046/j.1365-3032.1999.00114.x
- Kaneko, M., & Hall, J.C. (2000). Neuroanatomy of cells expressing clock genes in *Drosophila*: Transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian

pacemaker neurons and their projections. *Journal of Comparative Neurology*, 422, 66-94.
doi:10.1002/(SICI)1096-9861(20000619)422:1<66::AID-CNE5>3.0.CO;2-2

Kaneko, M., Helfrich-Förster, C., & Hall, J.C. (1997). Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: Newly identified pacemaker candidates and novel features of clock gene product cycling. *Journal of Neuroscience*, 17, 6745-6760.

Karpova, S., & Tomioka, K. K. (2006). Temperature entrainment of circadian locomotor rhythm in cricket (*Gryllus bimaculatus*) after removal of optic lobes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 145, 406-406.
doi:10.1016/j.cbpb.2006.10.019

Kostal, V., Zavodská, R., & Denlinger, D.L. (2009). Clock genes *period* and *timeless* are rhythmically expressed in brains of newly hatched, photosensitive larvae of the fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 55, 408-414.
doi:10.1016/j.jinsphys.2008.12.011

Lankinen, P., & Riihimaa, A. (1997). Effects of temperature on weak circadian eclosion rhythmicity in *Chymomyza costata* (diptera: Drosophilidae). *Journal of Insect Physiology*, 43, 251-260. doi:10.1016/S0022-1910(96)00094-7

Liu, T.X., Mahesh, G., Houl, J. H., & Hardin, P. E. (2015). Circadian activators are expressed days before they initiate clock function in late pacemaker neurons from *Drosophila*. *Journal of Neuroscience*, 35, 8662-8671. doi:10.1523/JNEUROSCI.0250-15.2015

- Majercak, J., Chen, W.F., & Edery, I. (2004). Splicing of the period gene 3'-terminal intron is regulated by light, circadian clock factors, and phospholipase C. *Molecular and Cellular Biology*, 24, 3359-3372. doi:10.1128/MCB.24.8.3359-3372.2004
- 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*. *Journal of Experimental Biology*, 218, 412-422. doi:10.1242/jeb.113233
- Mistlberger, R.E. (1994). Circadian food-anticipatory activity - formal models and physiological-mechanisms. *Neuroscience and Biobehavioral Reviews*, 18(2), 171-195. doi:10.1016/0149-7634(94)90023-X
- Miyazaki, Y., Goto, S. G., Tanaka, K., Saito, O., & Watari, Y. (2011). Thermoperiodic regulation of the circadian eclosion rhythm in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 57, 1249-1258. doi:10.1016/j.jinsphys.2011.05.006
- Myers, E.M. (2003). The circadian control of eclosion. *Chronobiology International*, 20, 775-794. doi:10.1081/CBI-120024214
- Parecis-Silva, P. V., Nuvoloni, F. M., & Feres, R. J. F. (2016). Day vs. night: The importance of the circadian cycle over metacommunities and predator-prey densities. *International Journal of Acarology*, 42, 141-148. doi: 10.1080/01647954.2016.1149220.

- Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 40, 1018–1029.
- Pittendrigh, C. S., & Bruce, V. G. (1959). Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In: *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. R.B. Withrow). *Publications of the American Association for the Advancement of Science*, No. 55, 475-505.
- Revel, F. G., Herwig, A., Garidou, M.L., Dardente, H., Menet, J. S., Masson-Pévet, M., Simonneaux, V., Saboureau, M., Pévet, P. (2007). The circadian clock stops ticking during deep hibernation in the European hamster. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 13816-13820. doi:10.1073/pnas.0704699104
- Rothenfluh, A., Young, M.W., & Saez, L. (2000). A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron*, 26, 505-514. doi:10.1016/S0896-6273(00)81182-4
- Rutila, J.E., Zeng, H.K., Le, M., Curtin, K.D., Hall, J.C., & Rosbash, M. (1996). The *tim*(SL) mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. *Neuron*, 17, 921-929. doi:10.1016/S0896-6273(00)80223-8
- Saunders, D.S. (1976). Circadian eclosion rhythm in *Sarcophaga-argyrostoma* - some comparisons with photoperiodic clock. *Journal of Comparative Physiology*, 110, 111-133.

Saunders, D.S. (1978). An experimental and theoretical analysis of photoperiodic induction in the flesh-fly, *Sarcophaga argyrostoma*. *Journal of Comparative Physiology* 124, 75-95.

Sehadova, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T., & Stanewsky, R. (2009). Temperature entrainment of *Drosophila*'s circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain. *Neuron*, 64, 251-266.

doi:10.1016/j.neuron.2009.08.026

Sehgal, A., Price, J.L., Man, B., & Young, M.W. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science*, 263, 1603-1606.

doi:10.1126/science.8128246

Shafer, O. T., Rosbash, M., & Truman, J. W. (2002). Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster*. *Journal of Neuroscience*, 22, 5946-5954.

Smith, P.H. (1985). Responsiveness to light of the circadian clock controlling eclosion in the blowfly, *Lucilia cuprina*. *Physiological Entomology*, 10, 323-336. doi:10.1111/j.1365-3032.1985.tb00054.x

Smith, R.F., & Konopka, R.J. (1981). Circadian clock phenotypes of chromosome aberrations with a breakpoint at the *per* locus. *Molecular & General Genetics*, 183, 243-251. doi: 10.1007/BF00270625

Smith, R.F., & Konopka, R.J. (1982). Effects of dosage alterations at the *per* locus on the period of the circadian clock of *Drosophila*. *Molecular & General Genetics*, 185, 30-36.

doi:10.1007/BF00333786

Tanaka, K., & Watari, Y. (2009). Is early morning adult eclosion in insects an adaptation to the increased moisture at dawn? *Biological Rhythm Research*, 40, 293-298.

doi:10.1080/09291010802067312

Tomioka, K., & Matsumoto, A. (2015). Circadian molecular clockworks in non-model insects. *Current Opinion in Insect Science*, 7, 58-64.

doi:http://dx.doi.org/10.1016/j.cois.2014.12.006

Veleri, S., & Wulbeck, C. (2004). Unique self-sustaining circadian oscillators within the brain of *Drosophila melanogaster*. *Chronobiology International*, 21, 329-342. doi:10.1081/CBI-120038597

Watari, Y. (2005). Comparison of the circadian eclosion rhythm between non-diapause and diapause pupae in the onion fly, *Delia antiqua*: The change of rhythmicity. *Journal of Insect Physiology*, 51, 11-16. doi:10.1016/j.jinsphys.2004.09.013

Watari, Y., & Tanaka, K. (2010). Interacting effect of thermoperiod and photoperiod on the eclosion rhythm in the onion fly, *Delia antiqua* supports the two-oscillator model. *Journal of Insect Physiology*, 56, 1192-1197. doi:10.1016/j.jinsphys.2010.03.022

- Weber, F., & Kay, S.A. (2003). A PERIOD inhibitor buffer introduces a delay mechanism for CLK/CYC-activated transcription. *FEBS Letters*, 555, 341-345. doi:10.1016/S0014-5793(023)01269-9
- Wheeler, D.A., Hamblen-Coyle, M.J., Dushay, M.S., & Hall, J.C. (1993). Behavior in light dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. *Journal of Biological Rhythms*, 8, 67-94. doi:10.1177/074873049300800106
- Winfree, A.T. (1970). Integrated view resetting a circadian clock. *Journal of Theoretical Biology*, 28, 327-&. doi:10.1016/0022-5193(70)90075-5
- Wuelbeck, C., Szabo, G., Shafer, O. T., Helfrich-Förster, C., & Stanewsky, R. (2005). The novel *Drosophila* tim(blind) mutation affects behavioral rhythms but not periodic eclosion. *Genetics*, 169, 751-766. doi:10.1534/genetics.104.036244
- Yocum, G. D., Rinehart, J. P., Yocum, I. S., Kemp, W. P., & Greenlee, K. J. (2016). Thermoperiodism synchronizes emergence in the alfalfa leafcutting bee (hymenoptera: Megachilidae). *Environmental Entomology*, 45, 245-251. doi:10.1093/ee/nvv152
- Yocum, G. D., Žďárek, J., Joplin, K. H., Lee, R.E., Smith, D.C., Manter, K.D., & Denlinger, D.L. (1994). Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *Journal of Insect Physiology*, 40,13-21. Doi: 10.1016/0022-1910(94)90107-4

- Yoshii, T., Fujii, K., & Tomioka, K. (2007). Induction of *Drosophila* behavioral and molecular circadian rhythms by temperature steps in constant light. *Journal of Biological Rhythms*, 22, 103-114. doi:10.1177/0748730406298176
- Yuan, Q., Metterville, D., Briscoe, A. D., & Reppert, S. M. (2007). Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Molecular Biology and Evolution*, 24, 948-955. doi:10.1093/molbev/msm011
- Žďárek, J., & Denlinger, D.L. (1995). Changes in temperature, not photoperiod, control the pattern of adult eclosion in the tsetse, *Glossina morsitans*. *Physiological Entomology*, 20, 362-366. doi:10.1111/j.1365-3032.1995.tb00827.x
- Zhan, S., Merlin, C., Boore, J. L., & Reppert, S. M. (2011). The monarch butterfly genome yields insights into long-distance migration. *Cell*, 147, 1171-1185. doi:10.1016/j.cell.2011.09.052