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Light and Temperature Entrainment of Two Circadian-Driven Behaviors in the Flesh Fly Sarcophaga crassipalpis

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Light and Temperature Entrainment of Two Circadian-Driven Behaviors in the Flesh Fly *Sarcophaga crassipalpis*

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Master of Science in Biology

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by

Raven Ragsdale

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Dr. Darrell Moore, Chair

Dr. Karl Joplin

Dr. Thomas C. Jones

Keywords: circadian rhythm, temperature entrainment, eclosion, locomotor activity, *Sarcophaga crassipalpis*, zeitgeber

ABSTRACT

Light and Temperature Entrainment of Two Circadian-Driven Behaviors in the Flesh Fly *Sarcophaga crassipalpis*

by

Raven Ragsdale

Circadian rhythms dictate the timing of both once-in-a-lifetime adult emergence (eclosion) and daily locomotor activity rhythms in the flesh fly *S. crassipalpis*. Light cycles are considered the primary environmental time cue (zeitgeber), but the life history of *S. crassipalpis* suggests that temperature cycles (thermocycles) may also play a key role. This work evaluates the efficacy of thermocycling as a zeitgeber in *S. crassipalpis*. We found that shifting both light and temperature cycles of sufficient amplitude affect the phasing of eclosion and locomotor activity, but result in different patterns. Additional experiments suggest greater thermocycle sensitivity during the late metamorphic period and that thermocycling reduces variance in eclosion times. Taken together, these findings suggest that temperature cycles can be used by *S. crassipalpis* to time eclosion and adult locomotor activity, and that *S. crassipalpis* may be physiologically primed to use thermocycle information during metamorphosis.

ACKNOWLEDGEMENTS

The author would like to thank her committee for the numerous hours spent in consideration of this work and the experiments described within. The author would also like to thank Marilyn Permenter, Colin Shone, Shae Crain, and numerous other unnamed fellow graduate and undergraduate students who voluntarily provided logistical and technical support.

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CHAPTER 1. INTRODUCTION

A huge diversity of organisms, from cyanobacteria to humans, exhibit circadian rhythms in a diversity of functions including metabolic processes, hormone secretions, the sleep-wake cycle, behavior, and even once-in-a-lifetime events such as eclosion (Saunders, 1973; Foster and Kreitzman, 2005). These rhythms are driven by endogenous biological clocks that cycle with periods close to 24 hours, approximating the length of a day on Earth. Circadian clocks enable organisms to anticipate environmental events (e.g., sunrise and sunset) which allows for the ecologically-appropriate scheduling of various biological processes. Circadian clocks are sensitive to daily environmental stimuli and remain in synchrony with the Earth's 24-hour day by continuously adjusting (entraining) to these signals (zeitgebers). Light is a well-known zeitgeber, but many other social and environmental inputs also cycle on a 24-hour basis and are important to include when considering circadian rhythms. Temperature as a zeitgeber has been evaluated in *Drosophila* (Yoshii et al., 2002; Glaser and Stanewsky, 2006; Tomioka and Yoshii, 2006; Currie et al., 2009), onion flies (Tanaka and Watari, 2003), honey bees (Moore and Rankin, 1993; Fuchikawa and Shimizu, 2007), cyanobacteria (Yoshida et al., 2009), isolated rat cells (Barrett and Takahashi, 1995), chick pineal gland cells (Herzog & Huckfeldt, 2003), in eclosion time in diapause *Sarcophaga crassipalpis* (Miyazaki et al., 2011), and in a limited number of other species. Soil temperature is a precise enough zeitgeber that it can be reliably predicted via computer model for a given location and date. Day-to-day temperature variation in soil at 5 cm, a depth similar to the depth *S. crassipalpis* burrow to pupate, is extremely dampened as compared to daily air temperature variation (Horton, 2012).

In *Drosophila melanogaster*, the gene *nocte* is required for temperature entrainment (Sehadova et al., 2009), and the clock proteins period and cryptochrome are suggested to be involved with the integration of temperature information (Boothroyd et al., 2007; Gentile et al., 2013). Further, specific areas of the *Drosophila* brain have been linked to temperature entrainment, separate from areas responsible for light entrainment (Miyasako et al., 2007). Though the overall changes which occur during metamorphosis are great on both a cellular and molecular level (Saunders and Zdarek, 1994; Hall et al., 2017), the circadian clock in *D. melanogaster* runs continuously from emergence as a first-instar larva through adulthood (Sehgal et al., 1992). In *S. crassipalpis*, a previous study has suggested that the circadian clock stops in early pharate adults and begins anew before reaching the late pharate adult stage (Short et al., 2016), but other evidence indicates that the circadian clock continues to cycle during this life stage (Joplin and Moore, 1999).

In addition to different genes, proteins, and neurons being responsible for entrainment in response to varying zeitgebers, there is also the consideration that different life stages or events may have unique rhythmic features and sensitivities. It has been proposed that wandering larval activity, diapause initiation, and eclosion are controlled by separate oscillators in *S. argyrostoma* (Saunders, 1986), and in *Drosophila*, it has been suggested that eclosion and locomotor activity are regulated by separate oscillators (Engelmann and Mack, 1978). Further, previous work has established that there is a critical time frame during early metamorphosis (days 1-9) after which *S. argyrostoma* is no longer able to alter eclosion timing in response to new light cycle information presented during metamorphosis (Saunders, 1979). No similar

work has been done to comprehensively describe if a similar sensitive period exists for eclosion or adult locomotor activity timing alteration in response to temperature cycles in *S. crassipalpis*.

The overall changes which occur during metamorphosis are great on both a cellular and molecular level (Saunders and Zdarek, 1994; Hall et al., 2017). In holometabolous insects, including *S. crassipalpis*, the body of the adult insect develops from a soft-bodied pupae with comparatively few noticeable external body parts into a complex and vastly changed adult. During this period of drastic development, the brain, nervous and sensory systems, digestive system, and all other adult features are developed anew or modified from existing larval systems (Hall et al., 2017). Immediately after metamorphosis, there are still additional steps to be taken to complete development. In flies, this includes emergence from the pupal casing followed by walking, air intake and pumping, and muscular contractions which allow for the fly to reach its final adult shape and size. Finally, wing expansion and hardening of the cuticle occur (Saunders and Zdarek, 1994).

The life cycle of *S. crassipalpis* makes this species a particularly useful model organism for evaluating temperature as a zeitgeber. Approximately 40% of *S. crassipalpis'* life cycle is spent undergoing a complete metamorphosis underground, where light signals are unlikely to penetrate the soil or the dark, sclerotized pupal casing (Saunders, 1986). Soil temperature is known to rise and fall over the course of the day (Carson, 1963), and therefore it could be ecologically advantageous for *S. crassipalpis* pupae to be especially sensitive to cycling soil temperatures to determine the time of day – a predictive ability that is important in determining the proper time to eclose, an

act which is a matter of life and death (Pittendrigh, 1954). An inappropriate eclosion time prevent appropriate wing development, as too early in the morning there is too much humidity for proper wing drying, and too late in the day it is too dry to allow the wings to fully expand before hardening. In both cases, this makes the newly-emerged fly an easy target for any passing predator, and any that may survive would almost certainly fail to find a mate (Pittendrigh, 1954). In addition to being high-stakes, the acceptable eclosion window is relatively small compared to the length of a day (Joplin and Moore, 1999).

With the current understanding of the function and general mechanism of the circadian clock from work with model species, and the evidence supporting how important circadian rhythms are to the living things which possess them (in *Drosophila*: Pittendrigh and Minis, 1972; in *Arabidopsis thaliana*: Dodd et al., 2005; in mammals: Wyse et al., 2010), there has been a surprisingly limited amount of work which seeks to investigate the ecological relevance of specific and varying zeitgebers and their perception at specific and varying developmental stages. This study seeks to investigate not only the effects of temperature as a zeitgeber in a poikilotherm, but also to investigate potential critical periods of temperature exposure during development.

This work will evaluate the ability of *S. crassipalpis* to utilize temperature information in relation to two different life events and three developmental periods within pupal metamorphosis. Previous studies (Yoshii et al., 2002; Tomioka & Yoshii, 2006; Currie et al, 2009; Miyazaki et al., 2011) have confirmed that temperature can inform eclosion time in some flies, including post-diapause *S. crassipalpis*, but locomotor activity as well as temperature cycle sensitivity throughout metamorphosis have been

much less intensively evaluated. The first question concerns the potential influence of light or temperature cycles on the circadian rhythms of eclosion and adult locomotor activity in flesh flies. While previous research has established that temperature cycles are able to dictate eclosion times in post-diapause *S. crassipalpis* (Miyazaki et al., 2011), the effects of temperature exposure on eclosion time in non-diapause flies and adult locomotor activity has not been recorded. One hypothesis proposes that adult locomotor activity rhythms do not necessarily mirror eclosion rhythms. It has been suggested that eclosion and adult activity may be controlled by separate oscillators (Engelmann and Mack, 1978; Saunders, 1979), and if each oscillator is optimized for use during specific life stages, each oscillator may be less or differently active during non-target life stages - potentially altering the timing of each life event in relation to one another. Additionally, as light and temperature reach their maximum values at different phases of the day (Carson, 1963), it is possible that locomotor activity patterns may vary based on the environmental stimuli provided. Alternatively, as light cycle information received during the wandering larval stage is retained through the pupal stage to control eclosion timing (Joplin and Moore, 1999), adult locomotor activity timing may be similarly regulated and informed, and therefore adult locomotor activity timing may be reasonably predicted from eclosion timing. This could be ecologically advantageous as pupae able to perceive current time cues to optimize eclosion time and adult locomotor activity simultaneously may be more immediately suited to adult life.

Further hypotheses can be made regarding specific periods in pupal development. As undergoing a complete metamorphosis requires many physiological

changes in a number of different stages (Denlinger & Zdarek, 1994; Hall et al., 2017), the varying internal composition of the fly may alter its sensitivity to external cues at various points. Though *S. crassipalpis*' internal clock continues to function throughout metamorphosis (Joplin & Moore, 1999), it may not be able to re-entrain to new environmental stimuli at all stages of development. Previous work suggests that key components of the molecular clock may only cycle at a reduced level during later stages of development (Short et al., 2016) and that pupae's ability to alter eclosion timing in response to novel light exposures decreases with more advanced stages in development (Saunders, 1979), indicating that earlier stages in pupal metamorphosis may be more sensitive to external stimuli than later stages. This supports the idea that eclosion and locomotor activity timing may be more completely entrained by exposure to temperature cycling during early pupal development rather than later exposures. Alternatively, as temperature entrainment may use a slightly different pathway than light entrainment (Gentile et al., 2013), temperature sensitivity may not mirror light sensitivity. Another possibility is that a limited-thermocycle-exposure period will not be a strong enough zeitgeber to completely override light cycle information presented during the wandering larvae stage, and there will be some effect of this previous light cycle information on eclosion and adult locomotor activity timing.

CHAPTER 2. MANUSCRIPT

TEMPERATURE ENTRAINMENT OF TWO DIFFERENT CIRCADIAN RHYTHMS IN THE FLESH FLY *SARCOPHAGA CRASSIPALPIS*

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Keywords: circadian rhythm, temperature entrainment, eclosion, locomotor activity, *Sarcophaga crassipalpis*, zeitgeber

1. Introduction

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In addition to varying zeitgebers, there is also the consideration that different life stages or events may have unique rhythmic features and sensitivities. It has been proposed that wandering larval activity, diapause initiation, and eclosion are controlled by separate oscillators in *S. argyrostoma* (Saunders, 1986), and in *Drosophila*, it has been suggested that eclosion and locomotor activity are regulated by separate oscillators (Engelmann and Mack, 1978). Further, previous work has established that there is a critical time frame during early metamorphosis (days 1-9) after which *S. argyrostoma* is no longer able to alter eclosion timing in response to new light cycle information presented during metamorphosis (Saunders, 1979). No similar work has been done to comprehensively describe if a similar sensitive period exists for eclosion or adult locomotor activity timing alteration in response to temperature cycles in *S. crassipalpis*.

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An additional hypothesis can be made concerning temperature compensation. Temperature compensation, the idea that organisms must be able to regulate the speed of their circadian clock even as environmental temperatures vary, has not been previously described in *S. crassipalpis*, but because temperature cycling is expected to be an effective eclosion signal in non-diapause *S. crassipalpis*, we expect to find that *S. crassipalpis'* free-running period lengths are very highly temperature compensated. If temperature fluctuations can cause circadian output, these same temperature fluctuations should not impair the function of the circadian system.

Further hypotheses can be made regarding specific periods in pupal development. As undergoing a complete metamorphosis requires many physiological changes in a number of different stages (Denlinger & Zdarek, 1994; Hall et al., 2017), the varying internal composition of the fly may alter its sensitivity to external cues at various points. Though *S. crassipalpis*' internal clock continues to function throughout metamorphosis (Joplin & Moore, 1999), it may not be able to re-entrain to new environmental stimuli at all stages of development. Previous work suggests that key components of the molecular clock may only cycle at a reduced level during later stages of development (Short et al., 2016) and that pupae's ability to alter eclosion timing in response to novel light exposures decreases with more advanced stages in development (Saunders, 1979), indicating that earlier stages in pupal metamorphosis may be more sensitive to external stimuli than later stages. This supports the idea that eclosion and locomotor activity timing may be more completely entrained by exposure to temperature cycling during early pupal development rather than later exposures. Alternatively, as temperature entrainment may use a slightly different pathway than light

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2. Materials and Methods

2.1 Experiments

For each experiment, 32 flesh fly pupae, *S. crassipalpis*, were sourced from the laboratory colony maintained by Dr. Karl Joplin at East Tennessee State University. Colony conditions were as follows: 15:9 LD cycle, 25° C, with photophase beginning at 0600 h each morning and concluding at 2100 h each night. Larvae for each experiment were collected on the day of larviposition and pupae were hand selected on the day of pupariation, so that all flies were the same age.

Each pupariated fly was placed into a 25mm diameter clear plastic tube containing dry sugar with access to water via an end cap attached to a length of clear plastic tubing (approximately 7.5 cm) filled with distilled water. A piece of cotton served as a filter between the water and the dry tube. These tubes were then loaded into an activity monitor (TriKinetics, Waltham, MA), which uses infrared beam interruptions to record activity levels. These monitors were then placed inside an incubator (Percival, Perry, IA), in which light and temperature conditions were programmed for the various

experiments. A temperature monitor (HOBO, Bourne, MA) was placed inside each incubator to record the temperature for the duration of each experiment.

For the first set of experiments, flesh flies were exposed to 12:12 thermophase:cryophase temperature cycles of four different amplitudes: 1°C, 2.5°C, 5°C, and 10°C (see appendix, supplemental fig. 1 for a visual depiction). All temperatures for all experiments were limited between 20°C and 30°C to maintain a suitable environment for the flies; previous experimentation demonstrated that temperatures below 20°C caused a reduction in activity while temperatures above 30°C markedly reduced flies' lifespans. The beginning of thermophase for each of these experiments was set to 1800 hours – 12 hours' difference from the onset of photophase the flies experienced as wandering larvae. The flies used for these experiments were placed into monitors the day of pupariation to maximize the amount of time each fly was exposed to the temperature cycling before eclosion. Temperature cycle exposure began approximately two weeks before eclosion, followed by 12 days of exposure after eclosion. In all trials, the temperature ceased cycling on the 13th day after eclosion, and afterwards held constant at their respective cryophase or thermophase temperatures (descriptive figure in appendix). This free running period was at least 7 days, or until the end of life for the majority of the flies. All of these experiments proceeded in constant darkness.

To investigate temperature sensitivity at different developmental stages, pupae from a single colony were collected the day of pupariation were placed in constant dark at 25°C and then exposed to four days of temperature cycling during early, middle, or

late metamorphosis. Temperature cycle exposure during days 1-4 of the pupal stage are referred to as the “early” group, exposure during days 5-8 comprise the “middle” group, and exposure during days 9-12 characterize the “late” group. For all groups, temperature cycles consisted of a 12:12 thermophase (25°C): cryophase (20°C) cycle with thermophase onset at 1800 h (12 hours offset from photophase experienced during the wandering larval stage). All days outside of the four-day temperature cycle exposure window were a constant 25°±1°C for all groups. Constant darkness was maintained for the duration of these experiments. For a visual representation of this experimental scheme, see fig. A.2 in appendix.

Four additional trials were performed for comparison purposes. During one of these trials, a group of flies was deprived of any light or temperature cues (DD, 25°C) from pupariation until the end of the experiment. During the second of these trials, light was administered at 1800 hours for a 12-hour period each day from pupariation until the end of the entrainment period (determined as described above). The temperature was held constant (25°C) for the duration of the experiment. The third trial simply took flies from the colony on the day of pupariation, placed them into monitors, and then returned them to colony conditions (25°C, LD 15:9, photophase onset 0600h). The fourth trial, LD 12:12 0600, exposed flies to an LD 12:12 light cycle with an onset time of 0600; this differed from the previous trial in that scotophase onset was moved to earlier in the subjective day. This trial also proceeded at 25°C. For each of these trials, eclosion times and locomotor activity were monitored as they were in the temperature-variant trials. Free running periods and experiment length were determined identically to all other experiments.

2.2 Data Analyses

For data analyses for all experiments, flies that did not survive more than five days after eclosion were only included in the eclosion analyses. Flies that did survive more than five days after eclosion but did not survive through the end of the experiment were included in all applicable data analyses sans the last 3 days of life, as many of the flies exhibited abnormal, continuous activity for these days.

Eclosion times for each fly were identified by finding the time of the first registered activity which was followed by at least one additional count in the following 5 minutes. These criteria were set to filter out erroneous activations which would not have accurately described eclosion timing. The time labels for each minute were coded such that the beginning of thermophase was set to ZT0, with every other minute relabeled in respect to this. Negative values indicate times before ZT0, positive values indicate times after ZT0. Deviation from the mean for eclosion times in each group was obtained by taking the absolute value of the difference between each fly's eclosion time and the average eclosion time for their respective group. These lists of values were then statistically compared, where appropriate, to compare the range of eclosion times among groups. Acrophase times and free running periodicity were selected using ACTi Metrics' Clock Lab circadian biology analysis software. Each of these values were taken from software-generated actograms or periodograms, respectively. Activity onset times were selected by determining the first registered activity for each day which was followed by additional activity within one hour. Onset times were chosen in this way as ClockLab-generated onset times were often not properly identifying an accurate onset

time (such as selecting a point not associated with any activity). Free-running period values were recorded using both chi-squared and Lomb-Scargle tests, and only flies with closely-agreeing scores from each test were used for any free-running period calculations. Average onset and acrophase times used in statistical analyses were derived from averaging values from the last four days of entrainment for each fly. For the groups which did not experience a true entrainment period, days 8-11 of life were used to match as closely as possible with the age of the flies in the entrained groups. These days were selected for these calculations as by this age all flies reached a stable phase relationship with their respective zeitgeber.

The data were determined to be non-normal overall using the Shapiro-Wilk test, and therefore all appropriate statistical comparisons were done using nonparametric tests. Mann-Whitney U tests, Kruskal-Wallis tests, and Friedman's test were used to make initial comparisons as described in the results section. Dwass, Steel, and Critchlow-Fligner's post hoc test (DSCF), Bonferroni-adjusted Wilcoxon, or Mann-Whitney U tests were used when appropriate. The significance criteria for each test was $p<0.05$.

3. Results

3.1 Light-dark cycle entrainment

3.1.1 Eclosion

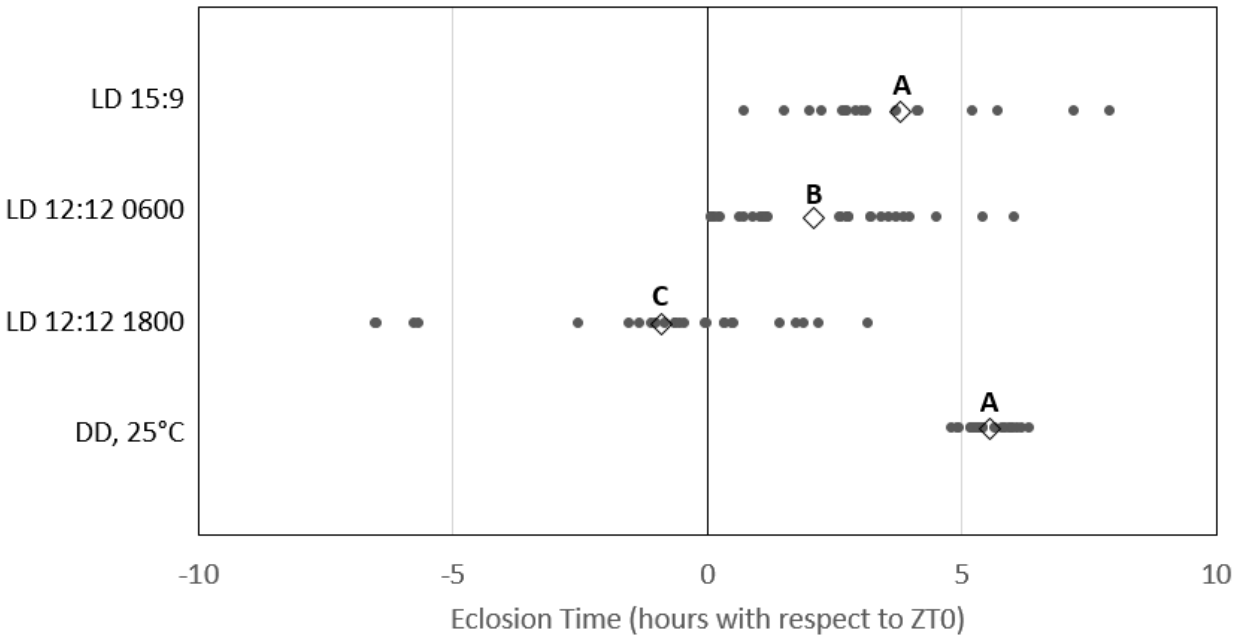

Figure 1: Eclosion times in light cycle exposed flies. Eclosion times shown in respect to ZT0. LD 15:9 denotes group held in colony conditions through metamorphosis and early adulthood (LD 15:9, photophase onset 0600, 25°C). LD 12:12 (0600) denotes the group exposed to LD 12:12, photophase onset 0600. LD 12:12 (1800) denotes the group exposed to LD 12:12, photophase onset 1800. DD, 25°C denotes the group kept in constant darkness at constant 25°C for the duration of the experiment.

Most light-exposed groups' average eclosion times occur after ZT0 with the exception of the LD 12:12 1800 group (fig. 1). All groups' eclosion times are significantly different from one another ($p \leq 0.0296$ for all pairwise comparisons) except for the LD

15:9 and DD, 25°C groups ($p=0.0596$). When comparing the variance of eclosion times among light-exposed flies, there is no significant difference among the LD 15:9, LD 12:12 1800, and LD 12:12 0600 groups ($p=1.0000$ in all cases). However, the variance in the DD, 25°C is significantly smaller than all other groups ($p \leq 0.0044$ for all comparisons).

3.1.2 Locomotor Activity

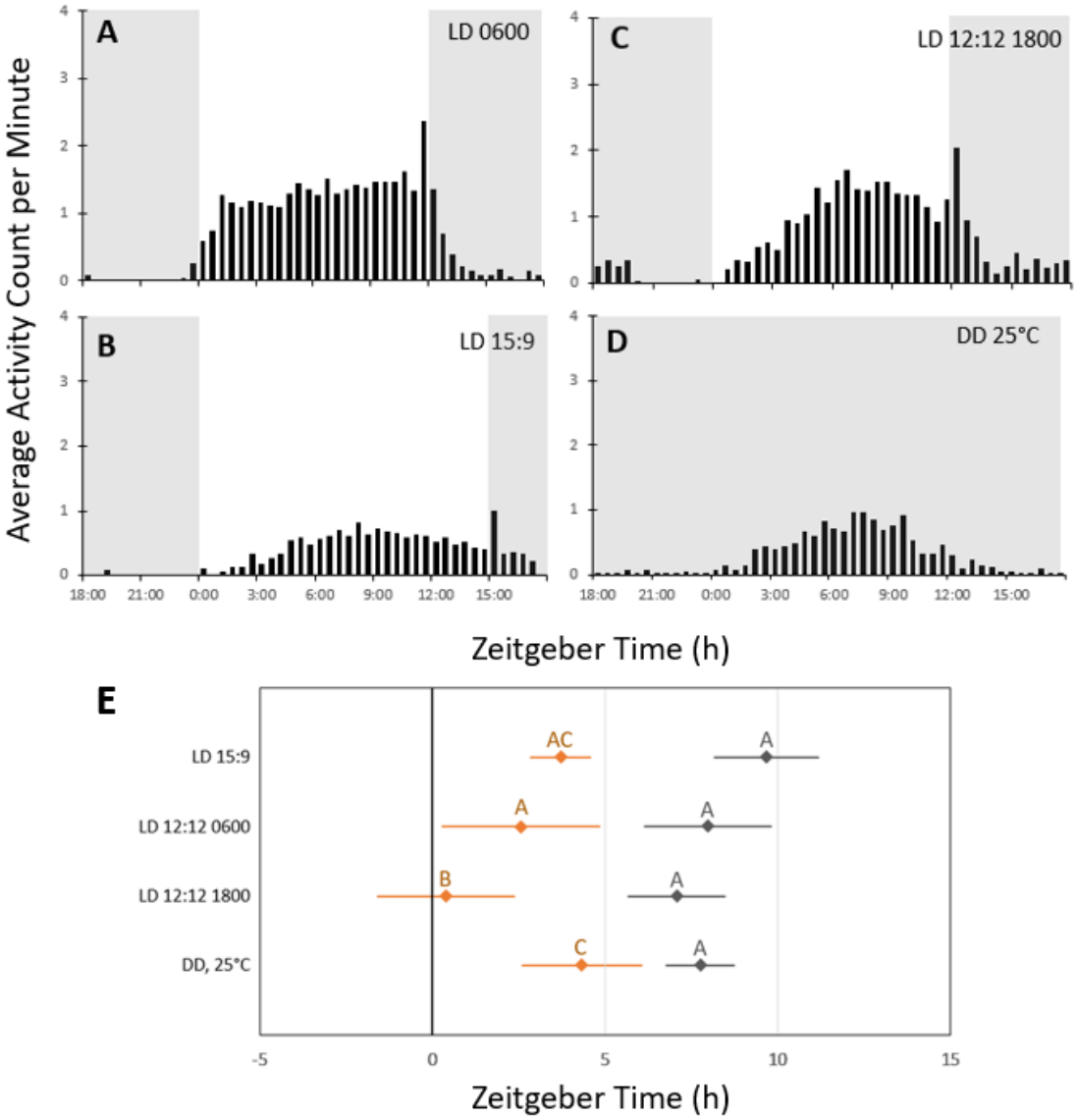

Figure 2: Locomotor activity in light cycle exposed flies. Panels A – D: black bars indicate average activity count per minute, averaged over 30-minute bins. Gray shading denotes scotophase. Panel E: Average acrophase and onset times in light cycle exposed adult flies. Onset times ± 1 standard deviation and significance are shown in orange. Acrophase times ± 1 standard deviation and significance are shown in gray.

Average activity onset times in all light-exposed groups occur after photophase onset (fig. 2). Onset times in the LD 12:12 1800 group are significantly earlier than onset times in all other groups ($p \leq 0.0402$ in all pairwise comparisons). Onset times in the DD, 25°C group are significantly later than onset times in the LD 12:12 0600 group ($p = 0.0036$). No other pairwise comparisons of onset time are significantly different. Average acrophase times occur later in the subjective day, approximately 7 to 9.5 hours after photophase onset. There were no significant differences in acrophase time for all pairwise comparisons of light-exposed flies ($p \geq 0.0786$ in all cases).

3.2 Temperature Entrainment

3.2.1 Eclosion

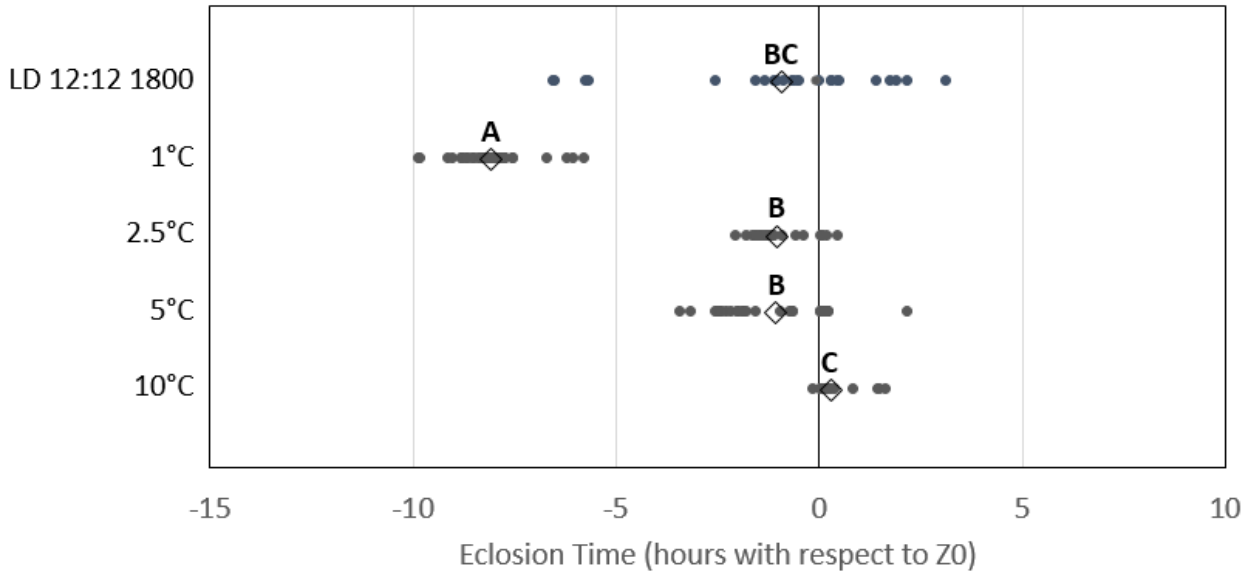

Figure 3: Eclosion times in thermocycle-exposed flies. Eclosion times shown with respect to ZT0 (approximately 1800h, photophase onset or thermophase onset by group). 1°C, 2.5°C, 5°C, and 10°C refer to the temperature amplitude for each of those groups. All temperature-exposed groups were held in constant darkness for the duration of the experiment. The “LD 12:12 1800” group was held at 25°C while experiencing an LD 12:12 light cycle. Lettering denotes significant differences in eclosion times.

When comparing thermocycle-exposed flies with light-dark cycle exposed flies, we see that, with the exception of the 1°C group ($p<0.0000$), there were no significant differences in eclosion times ($p=1.000$) (fig. 3). Within the thermocycle-exposed groups, the 2.5°C and 5°C amplitude groups’ eclosion times are not significantly different from one another ($p=1.0000$), but both of these groups eclose significantly earlier than the 10°C group ($p<0.0001$ for both comparisons). Again, all other thermocycle-exposed

groups are much later than the 1°C group ($p<0.0000$). In the LD 12:12 1800 group and the thermocycle-exposed groups with an amplitude of 2.5°C or greater, average eclosion times are extremely close to ZT0 – either in anticipation of thermophase onset or immediately after. The 1°C amplitude group exhibited eclosion times much earlier than all other groups. Eclosion variance is smaller in the thermocycle-exposed groups than in the LD 12:12 1800 group ($p<0.041$ or less) except for the 5°C temperature amplitude group, which is not significantly different from the LD 12:12 1800 group ($p=0.4902$).

3.2.2 Locomotor Activity

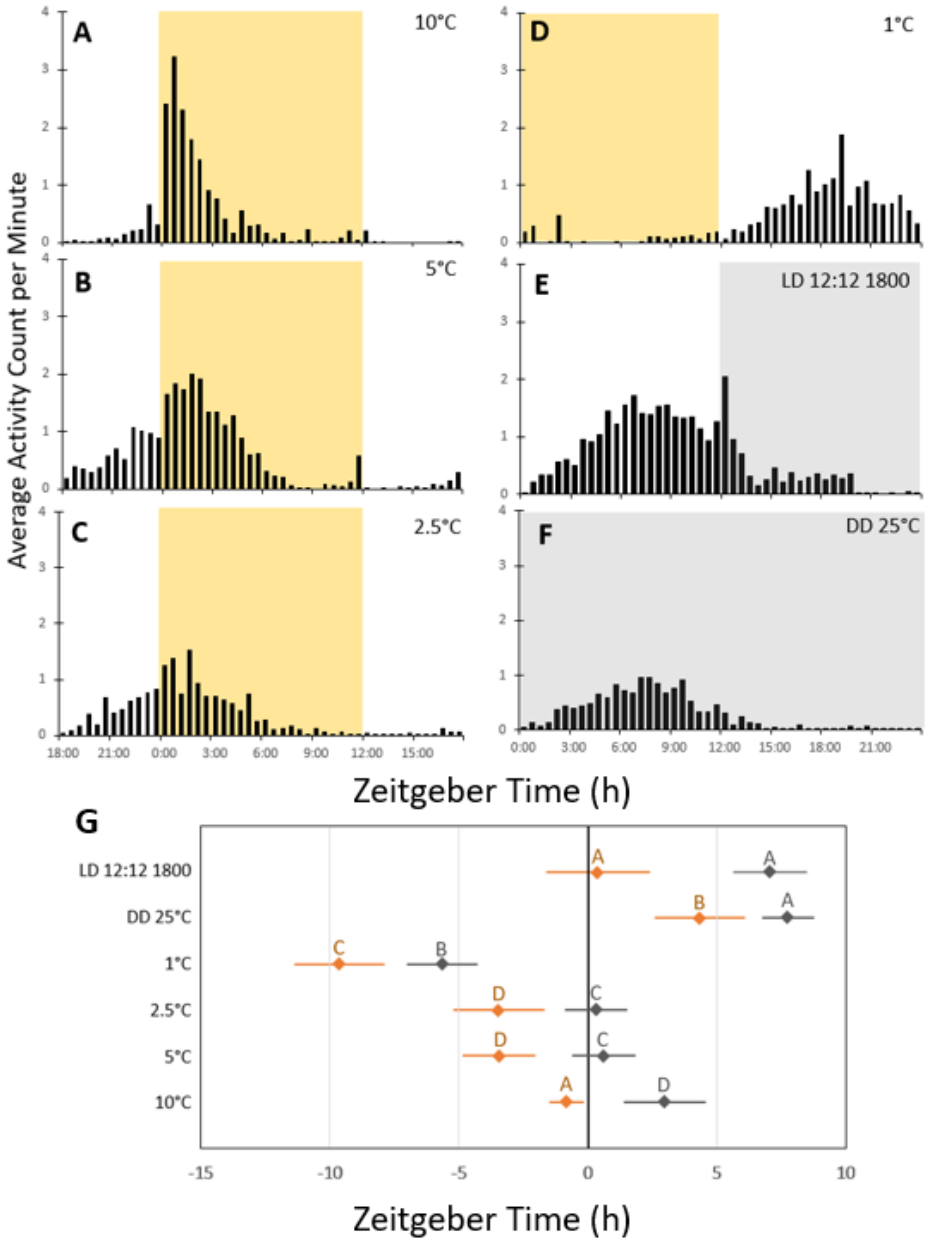

Figure 4: Locomotor activity in thermocycle-exposed adult flies. Panels A – F: black bars indicate average activity counts per minute, as averaged in 30-minute bins. For each group, denoted in the top right corner of each panel, flies' activity for days 8-11 of life were averaged. Yellow shading denotes thermophase. Gray shading denotes

scotophase. Panel G: Average acrophase and onset times in thermocycle exposed adult flies. Onset times ± 1 standard deviation and significance are shown in orange. Acrophase times ± 1 standard deviation and significance are shown in gray.

For all thermocycle-exposed groups with an amplitude of 2.5°C or greater, average activity onsets anticipate thermophase onset (fig. 4). Onset times for the 2.5°C and 5°C groups are not significantly different from one another ($p=1.0000$), but these groups' onset times are significantly earlier than the 10°C group ($p\leq0.0330$) and the 1°C ($p<0.0055$). Average acrophase times for all of these groups occur early in the subjective day. When comparing acrophase times, the 2.5°C and 5°C groups are not significantly different from one another ($p=1.0000$), but both of these groups' acrophase times are significantly earlier than the 10°C group ($p<0.0000$ and $p=0.0220$, respectively) and the 1°C group ($p=0.0044$ and 0.0088, respectively). The 1°C group's acrophase times are significantly later than the 10°C group ($p=0.044$. Overall, the 1°C amplitude group's average activity onset is much earlier than other thermocycle-exposed groups, and the average acrophase is much later in the subjective day – a pattern similar to what is seen in the LD 12:12 1800 and DD, 25°C groups.

3.3 Limited-Exposure Temperature Entrainment

3.3.1 Eclosion

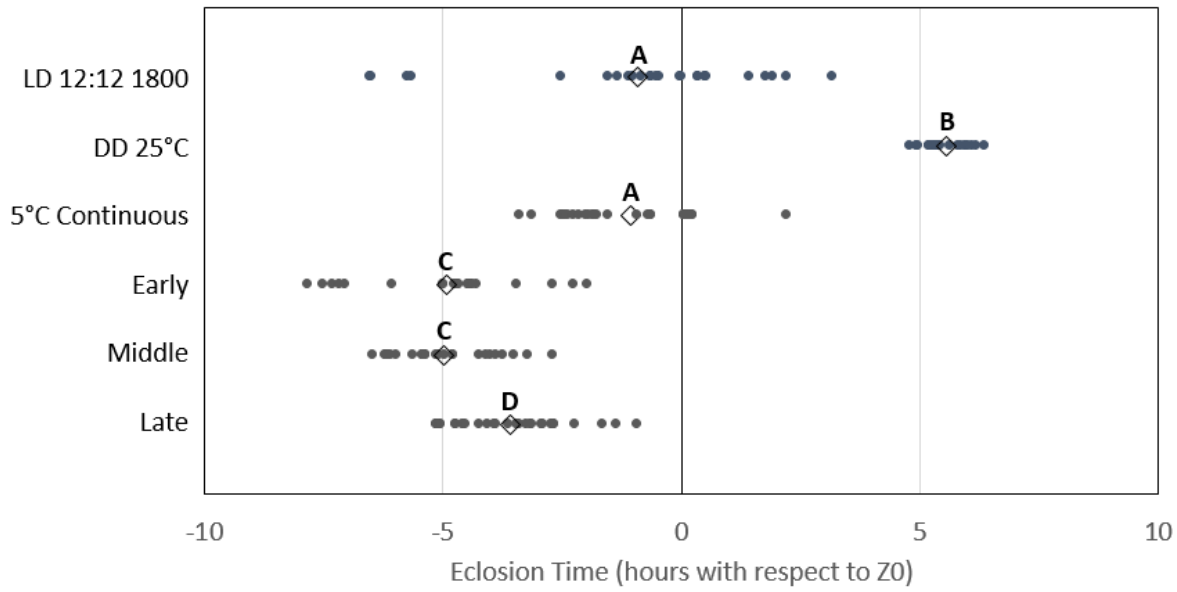

Figure 5: Eclosion times in limited-thermocycle-exposed flies. Eclosion times shown with respect to ZT0 (approximately 1800h, photophase onset or thermophase onset by group). The “LD 12:12 1800” group was held at 25°C while experiencing an LD 12:12 light cycle. The DD, 25°C was held in constant darkness at 25°C for the duration of the experiment. The 5°C group is the same 5°C group represented in fig. 1 (5°C temperature cycle amplitude). The Early Dev, Middle Dev, and Late Dev groups were exposed to 12:12 thermocycles with a 5°C amplitude during days 1-4, 5-8, or 9-12 of the metamorphic period (respectively).

Limited-exposure groups’ eclosion times aligned close to thermophase onset, but they did not achieve the same phase relationship with thermophase onset as the 5°C group which was exposed to thermocycling during the entire metamorphic period ($p<0.0000$ for all comparisons) (fig. 5). Eclosion also occurred significantly earlier than

the DD, 25°C group ($p<0.0000$), suggesting that the limited exposure to thermocycles nevertheless have some effect. Their eclosions also occurred significantly earlier than the LD 12:12 1800 group ($p<0.0001$ in all cases). When compared to one another, there is no significant difference in eclosion time between the early- and middle-exposure groups ($p=1.0000$), but both occur significantly earlier than the late-exposure group ($p=0.05$ and $p=0.0000$, respectively). It is interesting to note that early-, middle- and late-exposure groups' eclosion times exhibit greater variance than the DD, 25°C group ($p= 0.0203$, $p= 0.0035$, $p= 0.001$, respectively). There is no significant difference in variance among the three limited-exposure groups ($p=1.0000$ in all pairwise comparisons), and there is also no difference in variance when any of these three groups are compared to the LD 12:12 1800 group ($p\geq0.66$ in all cases) or the 5°C group ($p\geq0.59$ in all cases).

3.3.2. Locomotor Activity

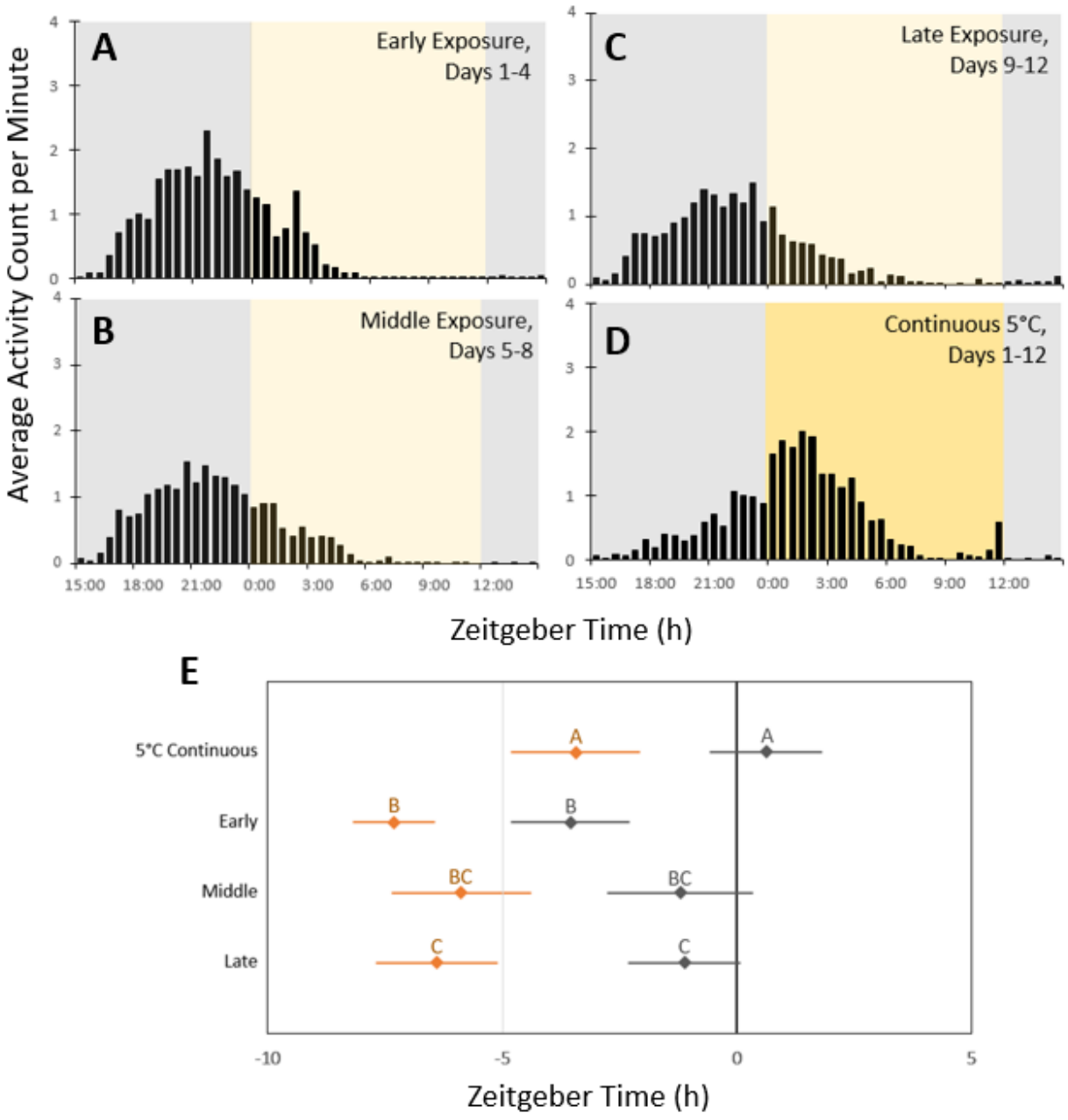

Figure 6: Locomotor activity in limited-thermocycle-exposed flies. Panels A-D: black bars indicate average activity per minute as average over 30-minute bins for each group. Yellow shading denotes thermophase; lighter yellow is used in groups with limited thermocycle exposure, darker yellow is used in the group with continuous

thermocycle exposure. Gray shading denotes scotophase. Panel E: Average acrophase and onset times in limited-thermocycle-exposure flies. Onset times ± 1 standard deviation and significance are shown in orange. Acrophase times ± 1 standard deviation and significance are shown in gray.

In all three limited-exposure groups, the average activity onsets anticipate the thermophase onset experienced during each group's exposure period and the average acrophase occurs early during the period corresponding the experienced thermophase. This is similar to the anticipatory activity onsets and early acrophase observed in the 5°C continuous-exposure group. However, the overall onset and acrophase times in the limited-exposure groups are significantly earlier than occur in the 5°C group (onsets: early: $p=0.0039$; middle: $p=0.0072$; late: $p=0.0024$; acrophase: early: $p=0.0012$; late: $p=0.0084$) (fig. 6). There is one exception to this pattern – the middle-exposure group's acrophase times are not significantly different from the 5°C group ($p=0.309$). Within these three groups, there are no significant differences in onset times among the early and middle or middle and late exposure groups to one another ($p=1.0000$ in both cases), but the early exposure group does become active earlier than the late exposure group ($p=0.0055$). The same patterns holds true for acrophase times; there is no significant difference in acrophase times when comparing the early and middle ($p=0.9735$) or middle and late exposure groups ($p=1.0000$), but acrophase times are significantly earlier in the early exposure group than in the late exposure group ($p=0.0275$).

3.4 Additional Comparisons

3.3.1 Eclosion

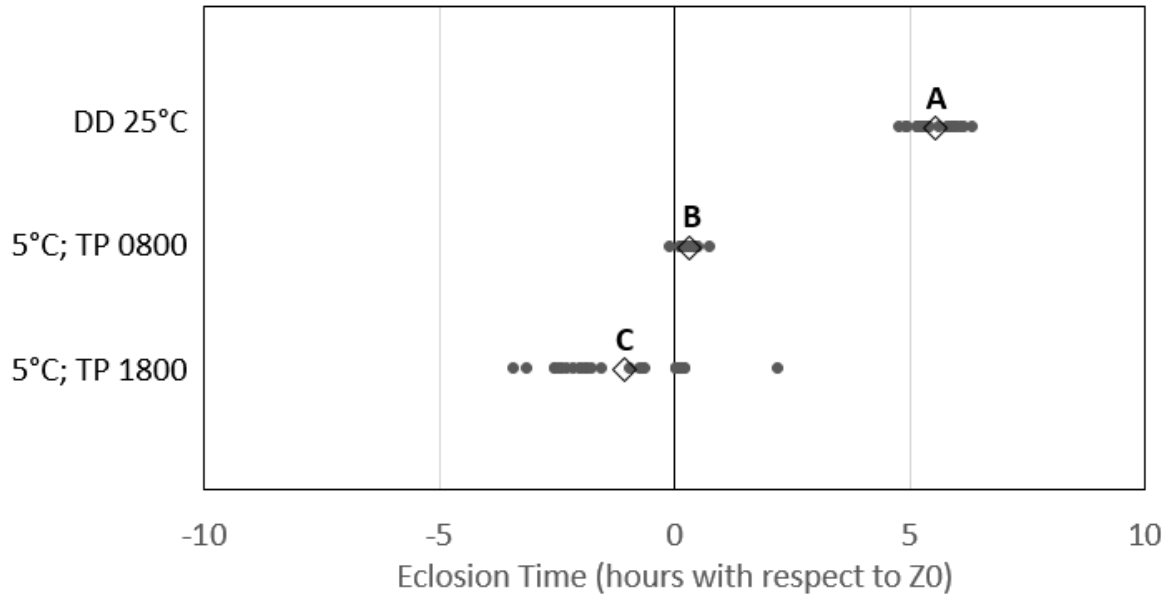

Figure 7: Eclosion times in three selected groups. DD, 25°C denotes the group held in constant darkness at 25°C for the duration of the experiment. 5°C, 0800 denotes the group which experienced a 5°C amplitude thermocycle with thermophase onset at 0800h. 5°C, 1800 denotes the group exposed to a 5°C amplitude thermocycle with thermophase onset at 1800h.

To better understand the reduced variance in eclosion times seen in the DD, 25°C group relative to all the other groups, we replicated the 5°C amplitude thermocycle experiment, positioning the thermophase onset at 0800 hours instead of 1800 hours. This 0800 start time was only two hours after photophase onset as experienced during the wandering larval stage rather than 12 hours in the 5°C 1800 hour group. If thermophase completely controls the phasing of eclosion, then we would predict that

the eclosion times would be 10 hours earlier in the 0800 start time group versus the 1800 start time group. The eclosion times did not follow this prediction, however, they did assume an earlier phase position – just not 10 hours earlier. Eclosion times differed among groups ($p<0.0001$ for all pairwise comparisons). The 5°C 1800 group's eclosion generally anticipates thermophase onset, the 5°C 0800 group's eclosion times are clustered immediately after thermophase onset, and the DD, 25°C group's eclosion times are several hours after ZT0 (fig. 7). The variance of eclosion times for each group were all significantly different from one another ($p<0.0001$ for all pairwise comparisons). The 5°C 0800 group has the smallest variance, followed by the DD, 25°C, then finally the 5°C 1800 group.

Additional comparisons were also made between the 1°C amplitude thermocycle group and the DD, 25°C group to better understand the phase position of eclosion in the 1°C group. Though comparing eclosion times with respect to ZT0 in these two groups lead to very different results, comparing the actual clock time of eclosion in the 1°C and DD, 25°C groups showed no significant difference ($p=0.083$). These results suggest that the 1°C thermocycle had no significant effect on the phasing of eclosion.

3.3.2 Locomotor Activity

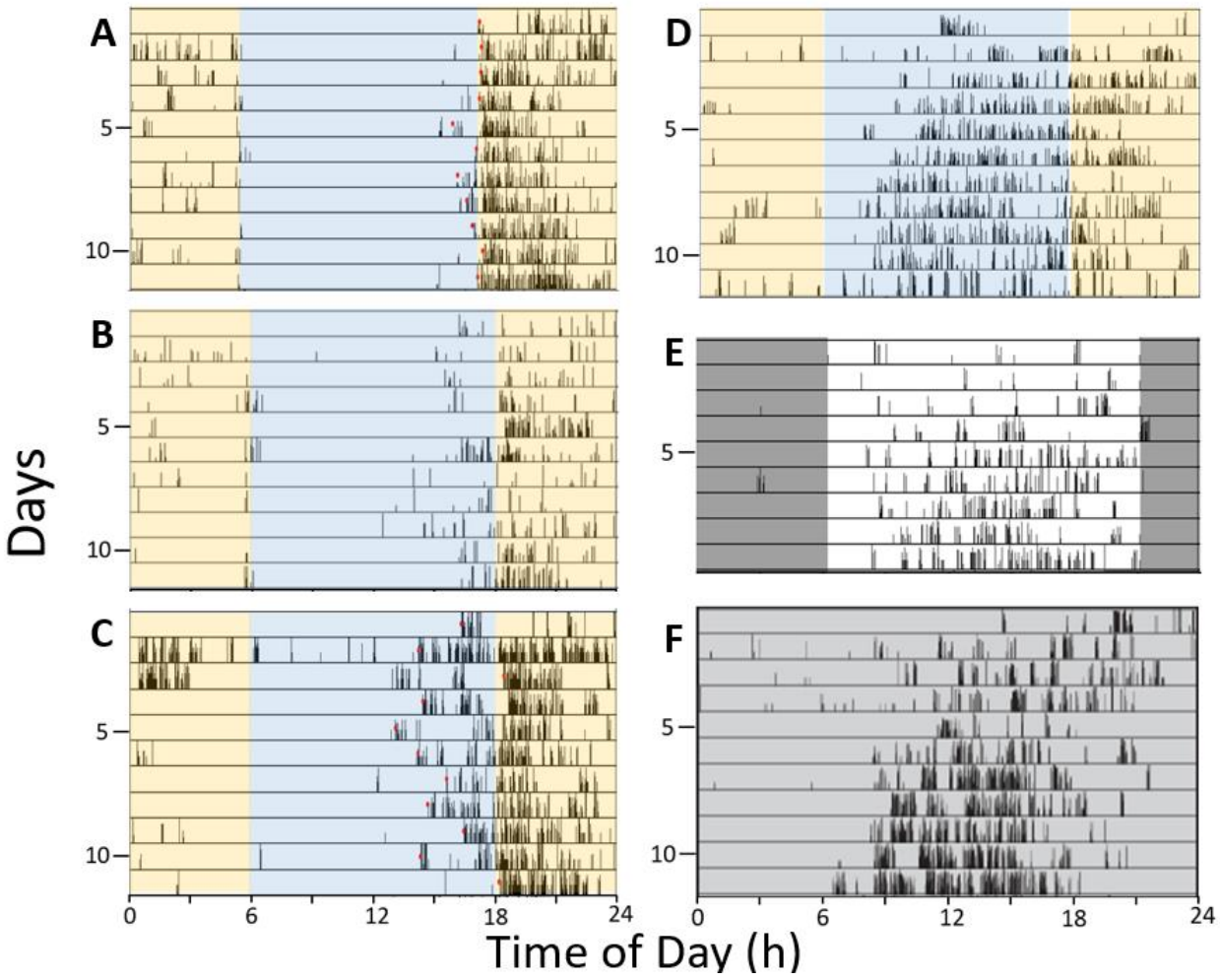

Figure 8: Representative actograms from thermocycle-exposed group and light-exposed groups. Panel A: 10°C; Panel B: 5°C; Panel C: 2.5°C; Panel D; 1°C; Panel E: LD 15:9; Panel F: DD, 25°C. Blue shading indicates cryophase. Yellow shading indicates thermophase. Gray shading indicates scotophase. No shading indicates photophase.

Similar to the findings for eclosion, the 1°C thermocycle had little or no effect on the phasing of locomotor activity (fig. 8). In the higher amplitude thermocycle-exposed groups, the bulk of locomotor activity occurs during thermophase and activity onset

anticipates thermophase onset by just a few hours. In the 1°C group, the bulk of locomotor activity proceeds during cryophase and activity onset is after cryophase onset. In the DD, 25°C group, the bulk of activity occurring during the period that correlates with photophase as experienced during the wandering larvae stage. Finally, in the LD 15:9 group, the bulk of activity occurs during photophase, with no anticipatory activity. Overall, the clock time of the activity periods in the 1°C, LD 15:9, and DD, 25°C groups are identical to one another.

3.5 Free-running Period

In all experimental groups, locomotor activity during constant conditions (DD, stable temperature) is virtually identical to locomotor activity during the last three days of entrainment. While there is a significant difference among free-running periods when all groups are compared to one another ($p=0.002$), there is only one pairwise comparison which is statistically significant (LD 12:12 0600 vs. Middle, $p=0.014$). The pooled average FRP is 23.81 ± 0.28h.

4. Discussion

4.1 Eclosion

As previously shown by Joplin and Moore (1999), *S. crassipalpis* pupae can retain light cycle information presented during their wandering larval stage throughout metamorphosis. When pupae are not exposed to either light or temperature cycling, they eclose over a 2-hour window in mid to late morning (as experienced during the wandering larvae stage). Exposure to a 1°C amplitude temperature cycle 12 hours offset from the light cycle experienced as wandering larvae for the duration of

metamorphosis produces eclosion times identical to the DD, 25°C group, indicating these flies are likely unable to perceive temperature fluctuations at or below 1°C. Every other group exposed to external time cues for the duration of metamorphosis exhibited eclosion times within a discrete window surrounding thermophase or photophase onset (fig.s 1, 3, 5, 7). These results are similar to those described in Miyazaki et al. (2011), with the major difference being that in the experiments described in the present work, eclosion time remained tightly gated in flies not exposed to a constant temperature, and we were unable to replicate the emergence pattern in response to a 1° amplitude square wave temperature cycle described therein. The difference in emergence timing after being held in constant conditions may be due to Miyazaki's use of post-diapause flies versus this study's use of non-diapause flies. One possible explanation could be that post-diapause flies are especially sensitive to temperature fluctuation as compared to non-diapause flies, as diapause termination is related to temperature (Denlinger, 1972).

The smallest eclosion window is seen in the 5°C amplitude group in which photophase onset occurred only two hours after photophase onset occurred during the wandering larval stage, followed in second place by the DD, 25°C group. This suggests that even though flies do not need additional timing information to reliably eclose, thermocycle information which agrees with previously-experienced light cycle information can reinforce previously-experienced time cues and serve to increase the accuracy of eclosion. One explanation for why the same effect is not seen in light-exposed flies may be because *S. crassipalpis* has not evolved to be especially receptive to additional light information during metamorphosis, as it is unlikely that they are

receiving any sunlight while under 5cm of soil, inside a thick and dark scleral casing (Saunders, 1986). Further, the 12-hour shifted thermocycle-exposed groups' eclosion time variances are comparable to that of the light-exposed groups; again, this supports the idea that cues that are not ecologically appropriate are not as easily compensated for by *S. crassipalpis*. In no natural environment would the cycle of night and day make a 180° switch within two weeks, so *S. crassipalpis* is simply not primed to compensate for this. Remarkably, even though these time cues are outside of the realm of what is possible in a natural environment, they are able to effectively and reliably shift eclosion time based on new timing information. Further, the windows of eclosion in response to these unnatural time cues are still comparable to what is typically seen in flies (Konopka and Benzer, 1971; Joplin and Moore, 1999). This further emphasizes the importance of proper eclosion timing and the physiological resources which must be devoted to accurately determining the best time for this high-stakes life event.

In respect to the eclosion times exhibited by the groups exposed to temperature cycling only during specific days of pupal metamorphosis, there is evidence to support a specially temperature-sensitive period of development during late metamorphosis. While exposure to temperature cycling within the first 8 days of pupal metamorphosis is able to inform eclosion timing, temperature cycle exposure during the last four days of pupal metamorphosis is able to shift eclosion time an additional 1.5 hours as compared to the other limited-exposure groups. This indicates that temperature cycle exposure at this time is able to more effectively and drastically change eclosion timing, even when the temperature amplitude and number of days of exposure are identical, suggesting an increased ability to assimilate temperature information during this sub-metamorphic

period. One speculative explanation for this could be that there is a reduction in the gross amount of clock proteins cycling during this stage of metamorphosis which weakens the molecular clock overall and therefore allows for the same zeitgeber to have a greater impact at this time than earlier in metamorphosis. It has previously been argued that a given zeitgeber should have a greater impact on the circadian clock in organisms with a weak oscillator as compared to those with a more robust oscillator (Winfree, 1970), and there is evidence that at least some clock proteins may be cycling at a reduced level during late metamorphosis in *S. crassipalpis* (Short et al., 2016). It is also interesting to note that this late-period sensitivity is precisely the opposite of what is seen with limited-period light exposure, where the critical period is early in metamorphosis in *S. argyrostoma*, another flesh fly (Saunders, 1979). Eclosion times in all groups exposed to temperature cycling only during a relatively short sub-metamorphic period were not shifted to match thermophase to the same extent that other temperature-exposed groups were, and this is likely due to the limited number of days of temperature. Each day of temperature cycling may only be able to adjust eclosion timing by a limited number of hours, meaning that more days of exposure to this cycling may be required in order for a 12-hour phase shift of eclosion time to be achieved. On the molecular level, this may be caused by direct interactions between heat and *per* mRNA. Previous work has established that heat exposure directly causes reductions in *per* mRNA in *Drosophila* (Sidote et. al, 1998), but of course this can only act on *per* mRNA that has already been produced or is in the process of being produced (as it is unclear whether this suppression of *per* mRNA is caused by destroying mRNA or preventing it from being produced). This necessarily limits the number of hours a

clock-driven behavior can be shifted in response to a single heat exposure. Since the limited-exposure flies are only receiving four days of introduced higher-temperatures, the limited number of hours eclosion has shifted may be reflective of the limited effect four heat exposures (versus 12) can have on the molecular clock.

4.2 Locomotor activity

Temperature cycling causes different behavioral patterns than light cycling. Light-exposed flies' activity gradually increases from photophase onset until reaching acrophase near the end of the subjective day, with no anticipatory behavior before lights-on (fig. 2). The DD, 25°C group and 1°C 12-hour thermophase shifted groups exhibit the same pattern when comparing their activity pattern to the light cycle they experienced as wandering larvae (fig.s 4, 8, 9). These results provide further evidence that *S. crassipalpis* pupae are able to retain light information experienced during the wandering larval stage, and that this information is preserved accurately enough to recreate light-appropriate locomotor patterns in the absence of any recognizable zeitgeber. All temperature-exposed groups which experienced a complete entrainment period exhibit activity in anticipation of thermophase, reach acrophase near the beginning of thermophase, and then gradually become less active as the day progresses (fig. 4). The discrepancy between the phase relationships of acrophase in light-exposed (or light-information-retaining) groups and temperature-exposed groups may be due to differences in phase relationships with peak light and peak temperature to time of day. In nature, peak soil temperature occurs several hours after the brightest part of the day. Exposure to light alone at a given time could activate behavioral programming that interprets the onset of photophase as subjective dawn. Exposure to

thermophase alone at a given time could activate behavioral programming which interprets thermophase onset as being several hours later in the subjective day. Both zeitgebers are able to entrain locomotor activity successfully, providing evidence that adult *S. crassipalpis* are able to perceive and use temperature information of a sufficient amplitude as they are light.

Groups which experienced temperature cycle exposure only during a four-day period during pupal metamorphosis exhibit an activity pattern that is overall similar to the other temperature-exposed groups, but which is shifted in relation to the thermophase onset they were exposed to during metamorphosis. For these groups, acrophase occurs significantly earlier than what would be expected if discrete temperature-exposure periods had the same effect as temperature cycles presented for the entirety of metamorphosis and beyond, but still different from onset and acrophase times experienced by light-exposed groups.

4.3 Free-running period

While for all groups the adult locomotor free-running period of *S. crassipalpis* is quite close to 24-hours, it is important to highlight that it is never equal to 24-hours. As discussed by Pittendrigh and Daan (1976), Hanson (1978), and Saunders (1986), a free-running period slightly different than 24-hours is required for optimal biological response to environmental stimuli – that is, period lengths different from 24-hours allow for entrainment. In a natural environment, the slight deviance of an organisms' clock from the exact length of a solar day on Earth would allow for the adjustments required to alter activity or metabolic patterns to occur more easily because having to slightly adjust one's clock with every new day ensures that the newest and most relevant

environmental information is being continuously integrated. A clock that is set to the most accurate time every day is more finely tuned than a clock that is set only once.

4.4 Conclusions

Eclosion and locomotor activity have different phase relationships with subjective time of day in non-temperature-responsive groups and groups exposed to limited days of temperature cycling only. While activity onset times do not perfectly mirror eclosion times, they are similar. There also seem to zeitgeber-specific locomotor activity patterns which are differ when thermocycles or light cycles are presented alone, but seem to reinforce one another when presented in a way that is ecologically appropriate. When considered alongside previous research which has suggested that the *per* and PER in *D. melanogaster* is directly acted upon by heat and light exposure (Boothroyd et. al, 2007), these observations suggest that eclosion and locomotor activity are controlled by one molecular oscillator which is sensitive to multiple time cues.

Eclosion has a phase position which cannot be directly predicted by the onset or offset of photoperiod experienced at any life stage. This emergence timing therefore could be a function of both photophase onset and scotophase onset during the wandering larval or pupal stages. Photo- or scotoperiod length could also potentially be a determining factor, as scotoperiod length has been established as the cause of diapause activation in flesh flies (Gnagey and Denlinger, 1984).

Overall, the data suggest that *S. crassipalpis* are able to use temperature information to inform eclosion timing as well as adult locomotor activity, though adult activity patterns do differ among flies with limited temperature cycle exposure, limited-

temperature-exposure groups, and light-exposed flies. Remarkably, flies either not exposed to temperature or light cycling or exposed only to low-level temperature cycling appear to retain very clear light cycle information obtained as wandering larvae which informs eclosion time, as described by Joplin & Moore (1999), as well as adult locomotor activity. Ecologically, this ability to perceive and use temperature information to set their internal clocks could be extremely useful to *S. crassipalpis* because of both the length of time spent underground (approximately 40% of their total lifespan) and the importance of emergence timing.

There is a period of pupal metamorphosis which is more sensitive to temperature cycling than other sub-metamorphic periods, but temperature cycle exposure at any point during metamorphosis is still able to affect eclosion and locomotor activity timing. Middle- and late-exposure flies' acrophase times are significantly later than early-exposure and adult-exposure flies' acrophase time, indicating that temperature cycle exposure at these times has a larger effect on adult locomotor activity timing. This supports the idea that flies are more sensitive or better able to use temperature cycle information during days 8-12 of pupal metamorphosis than flies earlier in pupal metamorphosis.

These findings emphasize the importance of temperature as a zeitgeber to flesh flies while also giving insight into circadian function during a complete metamorphosis. The difference in sensitive periods between light (as described in Saunders, 1979) and temperature (described herein) indicate that temperature and light support the idea that flies may be using slightly different pathways or proteins to alter eclosion timing (as described in Sidote et. al 1998 and Boothroyd et. al 2007). More research is needed to

fully understand the way temperature cycles are integrated into circadian systems, though it seems *per* and/or PER are heavily implicated. This study suggests there may be different degrees of importance placed on time-giving information dependent upon when it is presented in respect to the lifespan of the fly. Further, the effects of previously presented light cycle information seen in this work has implications for other circadian studies using lab-reared animals. Because there does seem to be an effect of previously presented light cycle information on *S. crassipalpis'* integration of new timing information, future studies should take careful considerations to mitigate or at least account for the rearing conditions of their experimental organisms. While this study does not provide a comprehensive view of every factor which could influence eclosion and locomotor activity patterns, it does present information which gives an introductory description of the effects of temperature on developing pupae as well as adult flies, and it raises questions related to gene expression and function during pupal metamorphosis in respect to the circadian system.

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APPENDIX: Supplemental Figures

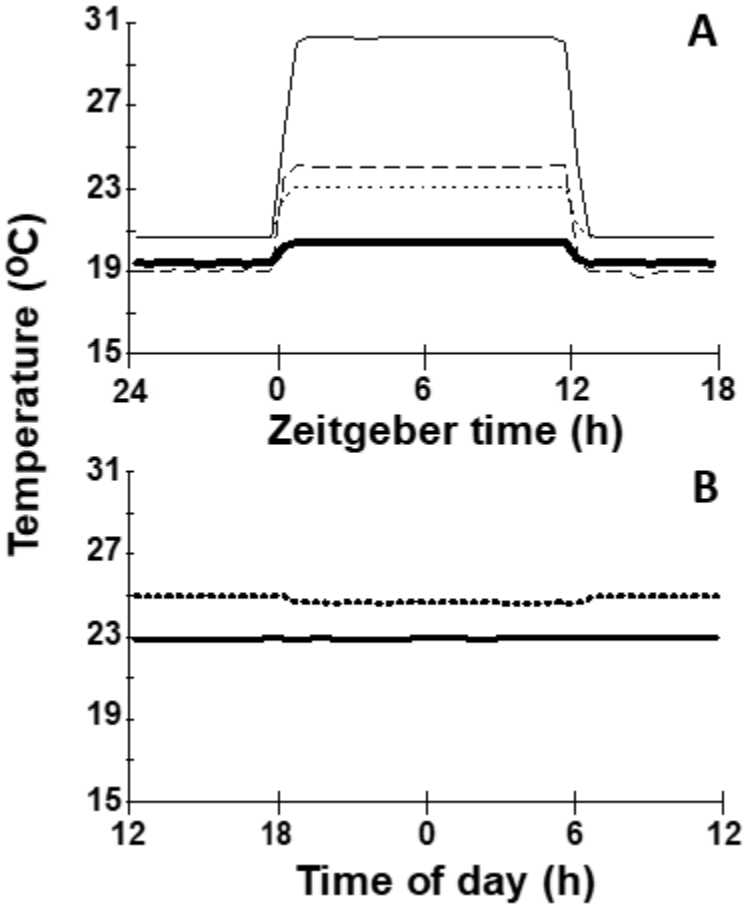

Figure A. 1: Thermophase and cryophase temperatures and phase position for all experimental groups. A) temperature cycle onset 12 after photophase onset during wandering larval light stage; bold line: 1°C amplitude, dotted line: 2.5° amplitude, dashed line: 5°C amplitude, thin line: 10°C amplitude; C) solid line: DD, 25°C temperature, dotted line: LD 12:12 1800.

Mann-Whitney U test comparing eclosion times			
Experimental Group		N	p(2)
DD, 25°C	By Sex	28	0.5823
LD 15:9	By Day	21	0.5419
	By Sex		0.2627
LD 12:12 (0600)	By Day	31	0.8729
	By Sex		
LD 12:12 (1800)	By Day	52	0.0703
	By Sex		0.1902
1°C	By Sex	26	0.6745
2.5°C	By Sex	29	0.332
5°C	By Day	29	0.749
	By Sex		0.8572
10°C	By Day	58	0.3222
	By Sex		0.2113
Early Dev.	By Day	21	0.177
	By Sex		0.0091*
Middle Dev.	By Day	25	0.2225
	By Sex		0.8337
Late Dev.	By Day	26	0.0111*
	By Sex		0.3173
Kruskal-Wallis test comparing eclosion times			
Experimental Group		N	p
1°C	By Day	26	0.7558

Table A. 1: The results of listed statistical tests comparing the indicated experimental groups' eclosion times by sex or by day of eclosion within the indicated group. * by statistical value denotes significant results. "LD 15:9" denotes the group monitored under colony conditions (LD 15:9, photophase onset 0600, constant 25°C). "LD" denotes the group exposed to 12:12 light:dark cycling. "DD, 25°C" denotes the group kept in constant darkness and at a constant temperature from pupariation. 1°C, 2.5°C, 5°C, and 10°C describe the temperature amplitude used for each listed group. "Early Dev" denotes the group which was exposed to 5°C amplitude temperature cycling only during days 1-4 of metamorphosis. "Mid Dev" denotes the group which was exposed to

5°C amplitude temperature cycling during days 5-8 of metamorphosis. “Late Dev” denotes the group which was exposed to 5°C amplitude temperature cycling during days 9-12 of metamorphosis.

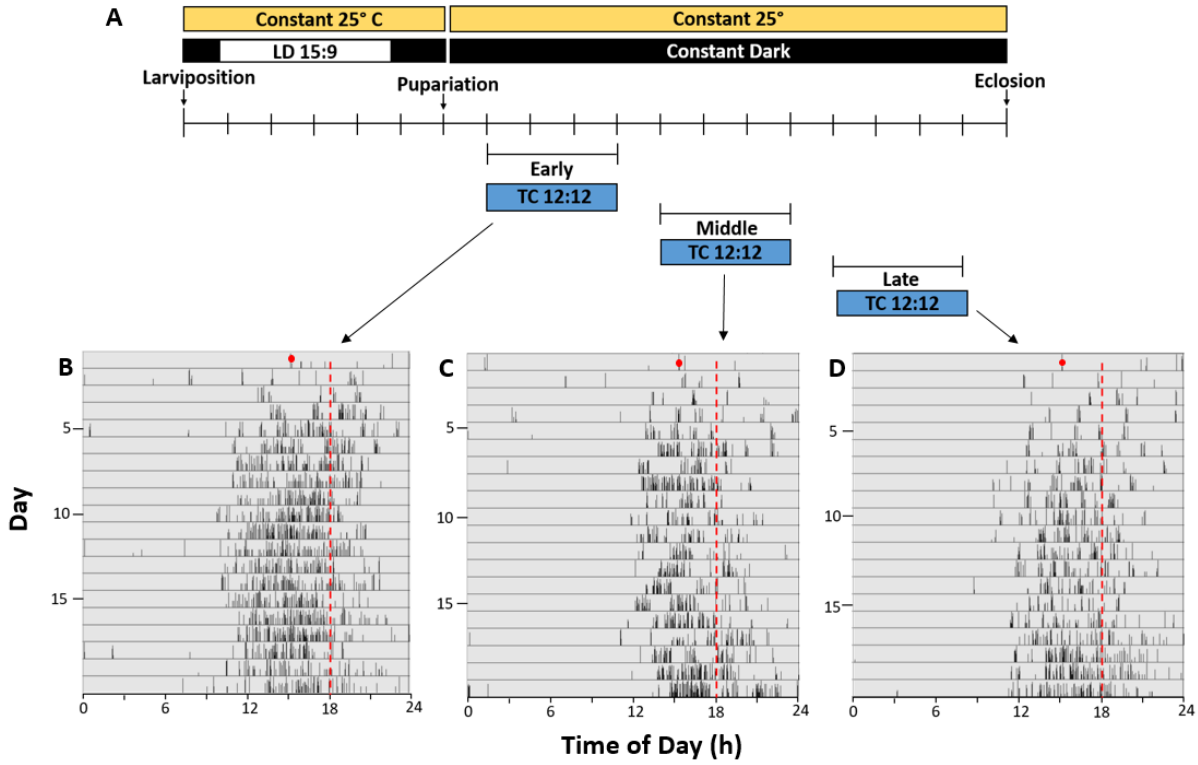

Figure A. 2: Experimental scheme and actograms for limited-thermocycle-exposed groups. Panel A describes the experimental design; larve developed under colony conditions (LD 15:9, photophase onset 0600, constant 25°C) until the day of pupariation and then were placed into constant dark at 25°C. Pupae were then exposed to TC 12:12 (20°-25°C, thermophase onset 1800) during the days 1-4 (early), days 5-8 (middle), **or** days 9-12 (late) of metamorphosis.Panel B depicts a representative actogram from the early-exposure group. Panel C depicts a representative actogram from the middle-exposure group. Panel D depicts a representative actogram from the late-exposure group.

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