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Comparing Behavior and Clock Gene Expression between Caterpillars, Butterflies, and Moths

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Abstract Circadian behavior is widely observed in insects; however, the mechanisms that drive its evolution remain a black box. While circadian activity rhythms are well characterized in adults within the order Lepidoptera (i.e., most butterfly species are day active, while most moths are night active), much less is known about daily activity and clock gene expression in the larval stage. Additionally, direct comparison of clock gene expression between day-active and night-active species reared together has not been quantified. Our study characterized the daily rhythms of caterpillar feeding and activity in addition to the gene expression of 2 central circadian clock genes, *period* (*per*) and *timeless* (*tim*), in larvae and adults of the day-active butterfly *Danaus plexippus* and the night-active moth *Heliothis virescens*. We found that neither *Danaus* nor *Heliothis* caterpillars are strictly diurnal or nocturnal like their adult counterparts; however, we found that slight rhythms in feeding and activity can arise in response to external forces, such as temperature and host plant. Expression levels differed between genes, between butterfly larvae and adults, and between butterfly and moth species, even though expression levels of both *per* and *tim* oscillated with a similar phase over 24 hours across all treatments. Our study, the first of its kind to investigate circadian timekeeper gene expression in 2 life stages and 2 species, highlights interesting differences in core clock gene expression patterns that could have potential downstream effects on circadian rhythms.

Keywords larval clocks, circadian oscillator, gene expression, *Heliothis virescens*, *Danaus plexippus*, *period*, *timeless*

Daily environmental cycles caused by the earth's rotation have driven the evolution of an internal timekeeping mechanism, the circadian clock, in almost all forms of life, including cyanobacteria, fungi, plants, and animals. In insects, circadian clocks synchronize rhythms of activity, sexual behavior, migration, calling, eclosion, oviposition, endocrine activity, and development (Saunders, 2002), which have increased their evolutionary success in diverse niches around the globe (Yerushalmi and Green, 2009).

Behavioral circadian rhythms in insects are ultimately programmed by a central timekeeper: a biochemical oscillator directed by cycling gene expression within specific neurons in the brain. In a

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negative feedback loop, the transcription of clock genes *period* (*per*) and *timeless* (*tim*)—and *cryptochrome-2* (*cry2*) in some insects such as butterflies and moths—is driven by the CLOCK (CLK)/CYCLE (CYC) heterodimer. Over 24 hours, PER, TIM, and CRY2 accumulate, dimerize, and eventually shut down their own transcription by binding to the CLK-CYC complex. A second feedback loop involves the genes *vrille, PAR-domain protein 1, clock*, and *cycle*. Kinases and other modulatory proteins keep the clock synchronized to environmental zeitgebers and running with a 24-hour period (e.g., the photoreceptor CRY1 degrades TIM to reset the clock). The oscillator is also paired to output pathways and clock-controlled genes that regulate the timing of biological rhythms throughout the organism.

Since the discovery of CRY2 in monarch butterflies (Yuan et al., 2007) and the increased availability of fully sequenced genomes (International Silkworm Genome Consortium, 2008; Zhan et al., 2011; Heliconius Genome Consortium, 2012; Cong et al., 2015a, 2015b, 2016a, 2016b), butterflies and moths (order Lepidoptera) have emerged as ideal model organisms in the intersection of circadian behavior, clock gene evolution, and gene expression. In the adult stage, circadian behavior is well characterized: nearly all adult butterflies are day active, while most moths are night active. Moreover, gene expression in the core timekeeping structure in the brain, the central oscillator, has been studied in a number of lepidopteran adults, including the monarch butterfly *Danaus plexippus*, Chinese silkmoth *Antheraea pernyi*, domestic silkmoth *Bombyx mori*, Mediterranean flour moth *Ephestia kuehniella*, and fall armyworm *Spodoptera frugiperda* (Reppert et al., 1994; Froy et al., 2003; Iwai et al., 2006; Zhu et al., 2008; Kobelkova et al., 2015; Hänniger et al., 2017). In these adults, *per* and *tim* oscillate over 24 hours, and transcript levels peak during the scotophase. However, very little is known of larval circadian rhythms. While certain caterpillar species have been observed as day active or night active in their native ranges (Leonard, 1970; Young, 1972; Stamp, 1977; Fitzgerald et al., 1988; Mcclure and Despland, 2010), it is unclear whether daily behavioral rhythms arise under laboratory conditions or are connected to core clock gene oscillation in the larval brain.

Environmental conditions such as temperature, photoperiod, and light intensity have all been suggested to affect *per* and *tim* expression amplitude and cycling (Pittendrigh et al., 1991; Iwai et al., 2006; Montelli et al., 2015). Therefore, direct comparison of gene expression between larvae and adults reared under the same conditions is essential for parsing differences in circadian clock gene expression. Controlling environmental variables that may affect expression also allows for comparison of oscillator function between adult butterflies and moths. Such a comparison could highlight interspecific differences in clock gene oscillation that may drive their opposite activity rhythms. Similar clock gene expression patterns between butterflies and moths, on the other hand, would confirm that diurnal and nocturnal activity rhythms evolved from functional mechanisms separate from the oscillator.

It has been argued that divergence in circadian timing is always caused by downstream mechanisms rather than functional changes to the oscillator itself (Tomioka and Matsumoto, 2015). However, this hypothesis has been challenged by a recent study of 2 *S. frugiperda* moth strains that have recently diverged onto different host plants and have evolved different circadian mating rhythms. Using quantitative trait locus analysis, Hänniger et al. (2017) identified a candidate gene within the oscillator, the clock gene *vrille* that regulates *clk* transcription, responsible for differential timing between strains, and showed that *vrille* expression was higher in one strain than the other. Additionally, core clock genes are rapidly evolving within Lepidoptera. Clock genes *per* and *tim* are highly divergent "speciation hotspots" between sister species of both hairstreak and tiger swallowtail butterflies (Cong et al., 2015b, 2016b). Across 26 species of butterflies and moths, only 14% of alignable amino acid residues remained invariant in the *per* gene, and *per* amino acid substitutions occurred more than 40 times faster than in the nuclear housekeeping gene *elongation factor 1-α* (Regier et al., 1998). Whether this molecular evolution accompanies functional changes to the oscillator that affect downstream rhythms remains an unsolved question.

Our study explores the daily rhythms and underlying circadian oscillator function between 2 life history stages (larva and adult) of the diurnal butterfly *D. plexippus* and the nocturnal moth *Heliothis virescens*. We first examined larval behavior in a series of experiments designed to test whether caterpillars exhibit a daily rhythm of feeding and activity and whether behavior changes in different abiotic and biotic conditions. We then quantified the daily oscillation in *per* and *tim* mRNA levels in both larvae and adults of both species to identify intraspecific and interspecific differences in oscillator function that may play a role in the evolution of circadian behavioral rhythms.

METHODS

Insects

The monarch butterfly *D. plexippus* and the tobacco budworm moth *H. virescens* were used in caterpillar

behavior and gene expression experiments. *Danaus* larvae and adults were the laboratory-reared offspring of butterflies obtained from the Tarifa area in southern Spain. *Danaus* larvae were reared on *Asclepias curassavica* plants in the greenhouse at the University of Amsterdam. Butterflies were reared in mesh cages and were provided with sponges soaked in 10% honey water that were replaced daily. Populations of *Heliothis* moths have been laboratory reared at the University of Amsterdam since 2011 and originate from North Carolina, where eggs were first collected from the field in 1988, supplemented with new field collections over the years, and reared at the Max Planck Institute for Chemical Ecology since 2003. Larvae were cultured in rearing cups containing a pinto bean–based "artificial" diet. Upon eclosion, moths were given a small cotton roll saturated in 10% sugar water.

Larval Feeding and Behavior

Feeding rhythm of Danaus larvae. Two experiments quantified the timing of *Danaus* caterpillar feeding. *Danaus* caterpillars were reared on *A. curassavica* plants at the University of Amsterdam for 4 to 5 generations prior to experimentation. In the first experiment, individual third-instar and fourth-instar caterpillars were placed in Petri dishes with a detached *A. curassavica* leaf. The dishes were kept in a temperature-controlled climate chamber under a 16:8 light:dark (L:D) rhythm at 25 °C and 60% relative humidity (n = 10). Each leaf was photographed at 6 time points during the 24-hour experiment at zeitgeber time (ZT; i.e., hours after the onset of photophase) 9, ZT 12.75, ZT 16, ZT 0, ZT 4, and ZT 9 (i.e., hour 24 of the feeding trial). Herbivory was quantified per individual caterpillar as the reduction in leaf surface area over the length of the experiment, measured in ImageJ version 1.49 (http://imagej.nih.gov/ij). In a second experiment, the same protocol was repeated in a greenhouse with the same L:D rhythm, where the temperatures were cooler at night (n = 8). Daytime greenhouse temperatures ranged from 24 °C to 27 °C and dropped to a nighttime average of 22.5 °C, with a low of 21.5 °C. Leaves were photographed at ZT 13, ZT 16.5, ZT 2.5, ZT 6.25, ZT 9.5, and ZT 13. Larvae that molted during data collection were not included in the analysis.

In the above experiments, feeding rhythms per caterpillar replicate were first calculated as the percentage of total daily feeding at each time point over 24 hours; thus, the extent of feeding at hour 0 (i.e., 0% feeding extent) and hour 24 (i.e., 100% feeding extent) was set to be identical across all caterpillar replicates. Deviation in the feeding rate was then calculated as the percentage feeding at each time point subtracted by the expected no-change feeding value to produce a time series of percentage change that was additive for each replicate (i.e., $\text{observed}_{t1} - \text{expected}_{t1}$). The resulting time series therefore begins and ends with zero-deviation values, as expected and observed feeding extent is equal at t = 0 hours and t = 24 hours. One-sample t tests were used to determine whether the deviation at each time point differed from the null hypothesis of a constant feeding rhythm (i.e., feeding deviation = 0). Kruskal-Wallis tests were used to compare feeding deviation values between time points of one series as well as between experiments.

Feeding rhythm of Heliothis larvae. An additional experiment quantified the daily feeding rhythm of *Heliothis* larvae. Each caterpillar was placed in an individual rearing cup of an artificial diet in a temperature-controlled climate chamber (25 °C) under a 16:8 L:D rhythm (n = 8). At each time point over 24 hours (ZT 8.3, ZT 11.8, ZT 15, ZT 0, ZT 4.7, and ZT 8.3), the caterpillar and frass were removed from the cup, and the diet was weighed. The caterpillar was then placed back into its own cup for the duration of the experiment. Four control cups of diet were also weighed at each time point to account for mass loss due to evaporation. Feeding extent was quantified for every caterpillar by subtracting replicate cup mass loss by the average control cup mass loss for each time point. Larvae that molted were not included in the analysis. Deviation in the feeding rate was calculated as above.

We also directly observed *Heliothis* caterpillar feeding behavior during 2 greenhouse experiments in December 2016 and April 2017. In December, *Heliothis* caterpillars were monitored for 24 hours on either *Nicotiana attenuata* plants or on an artificial diet in a greenhouse under a 17:7 L:D rhythm with a fluctuating temperature that was cooler at night. Caterpillars were placed on *N. attenuata* plants in the greenhouse 4 days before experiment onset, and at the start of the experiment, each caterpillar was separated onto individual plants (n = 25). Caterpillars on the artificial diet were separated into individual plastic breeding cups half-filled with a pinto bean diet and closed with a paper lid (n = 16). Individual caterpillars were observed for 30 seconds each hour for 24 hours and were marked as either "feeding" or "not feeding" based on observation of mandible movements. At night, caterpillars were observed using a red-light headlamp. We saw no indication that the red light affected caterpillar behavior. In April, *Heliothis* caterpillars were observed on either *Nicotiana benthamiana* or *Zea mays* plants, this time in a greenhouse under a 16:8 L:D rhythm. Caterpillars that fell or moved off their individual plant were

replaced with a new caterpillar from the laboratory stock (n = 14 caterpillars on n = 12 *N. benthamiana* plants and n = 26 caterpillars on n = 13 *Z. mays* plants over 24 hours). Each caterpillar was observed for 30 seconds every 30 minutes for 24 hours and marked as feeding or not feeding. Replicates that molted during our observations were excluded from the analysis.

In both experiments, a temperature probe recorded the drop in temperature at night; however, only the average daytime versus nighttime temperature was recorded in April. In December, the temperature was an average of 28.6 °C during the day and 20.4 °C at night, with the peak daytime temperature reaching 31.5 °C (Fig. 2A), while in April, average temperatures were 21.4 °C during the day and 18.0 °C at night (Fig. 2B).

The proportion of feeding caterpillars per time point was tallied and 1-way analyses of variance (ANOVAs) performed for proportional differences between day and night. Generalized estimating equations (GEEs; "geepack" package in R) with a binomial distribution and an autoregressive correlation structure were used to test whether ZT or phase predicted feeding, taking into account within-caterpillar and between-caterpillar variance. The effect of treatment was also tested by comparing GEE models with and without treatment terms using Wald statistics. We also used the "cosinor" package in R to fit a cosine curve to each 24-hour time series, whereby a significant p value for the parameters amplitude (height from mesor to peak) and acrophase (time of peak feeding) would indicate a sinusoidal oscillation in feeding.

Activity rhythm of Heliothis caterpillars. During our observational experiments in December 2016 and April 2017, each caterpillar replicate was also marked "active" or "not active" based on any observable movement including walking, searching, and feeding. Caterpillar activity over 24 hours was analyzed using 1-way ANOVA, GEE modeling, and cosinor modeling as described above.

Clock Gene Expression in Larvae and Adults

Gene expression patterns in larvae. To quantify the expression of 2 core circadian clock genes—*period* (*per*) and *timeless* (*tim*)—over 24 hours, we conducted a real-time quantitative polymerase chain reaction (RT-qPCR) experiment with mRNA from the heads of *Danaus* larvae feeding on *A. curassavica* and *Heliothis* larvae feeding on *N. attenuata*. Four mated *Danaus* females, collected from Spain in early March 2016, laid eggs on *A. curassavica* plants, and their offspring were collected for the RT-qPCR experiment (i.e., we sampled from 4 sets of siblings). The parents of the sampled *Heliothis* larvae laid eggs on cheesecloth that was transferred to *N. attenuata* plants in the greenhouse within 1 day of first hatching. All caterpillars were sampled in their third instar on their respective host plant.

Danaus larvae were collected every 2 hours for 24 hours, starting at ZT 4, and *Heliothis* larvae were collected every 2 hours starting at ZT 5. Sampled individuals were flash frozen in liquid nitrogen at each time point and then stored at −80 °C. Sampling took place in March 2016. Individuals from all treatments were reared in the same greenhouse in a 16:8 L:D rhythm.

Primers for target genes *Dp per, Dp tim, Hv per*, and *Hv tim* were creating using Primer3 web version 4.0.0 (http://bioinfo.ut.ee/primer3) and tested by PCR with Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific). Reference genes for each species were chosen based on their expression stability over time and specifically for their strength as a reference gene across lepidopteran developmental stages (Pan et al., 2015; Liu et al., 2016). The sequence for the *Danaus* reference gene *elongation factor 1-α* was obtained from Pan et al. (2015) (Suppl. Table S1).

Heads were removed from frozen individuals with a razor blade and were ground in individual epitubes with a sterile pestle in liquid nitrogen. RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research) and was treated with DNase using the Ambion Turbo DNase kit (Thermo Fisher Scientific). RNA was diluted to 25 ng/μL before reverse transcription using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR was conducted using HOT FIREPol EvaGreen qPCR Mix Plus Rox (Solis BioDyne) with 2 μL cDNA per reaction. The reaction ran at 50 °C for 2 minutes, 95 °C for 15 minutes, and then 40 cycles at 95 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds, followed by the melting curve analysis.

Unscaled expression levels were calculated as the number of target gene molecules per number of reference gene molecules, assuming 100% primer efficiency (confirmed from a dilution series that supported efficiencies of 100% or slightly higher). Then, unscaled expression values within each treatment were scaled to the highest value between *per* and *tim*, so that maximum expression was essentially set to 1 for *Danaus* larvae and *Heliothis* larvae, respectively. Gene expression differences were tested in R version 3.3.1. Due to data nonnormality (confirmed with the Shapiro-Wilk test), we relied on Kruskal-Wallis testing to compare expression within and between whole time series. We also used the "cosinor" package in R to fit a cosine curve to each expression time series (scaled to the highest expression value of

the treatment) to test whether transcript levels oscillated with a significant amplitude and acrophase.

Comparing Heliothis caterpillars on a plant diet versus artificial diet. To examine whether plant feeding affected the expression of *per* or *tim*, we also sampled *Heliothis* larvae that were reared on an "artificial" diet, following the same sampling procedures described above for *Heliothis* caterpillars collected from *N. attenuata* plants. Diet-reared caterpillars were separated into individual rearing cups prior to collection and were flash frozen in liquid nitrogen every 2 hours for 24 hours, beginning at ZT 4. Transcript levels of *per* and *tim* were quantified using the RT-qPCR method described above.

per and *tim* expression was measured as the number of target gene molecules per number of reference gene molecules, and these values were scaled to the highest single expression value across both plant-reared (on *N. attenuata*) and diet-reared *Heliothis* larvae. Kruskal-Wallis tests analyzed the expression level change over 24 hours in diet-reared larvae as well as expression differences between plant-reared and diet-reared larvae. Cosinor models were also fit to diet-reared *per* and *tim* time series to examine whether gene expression oscillated robustly.

Gene expression patterns in adults. The expression of *per* and *tim* over 24 hours was also quantified in *Danaus* and *Heliothis* adults. Adults of both species pupated, eclosed, and were ultimately sampled in April 2016 in the same greenhouse from which larvae were also collected. Virgin adults were collected, 5 to 6 days after eclosion, every 2 hours for 24 hours beginning at ZT 4. The sex of each adult was recorded. *Danaus* adults were sampled from the same 4 sets of siblings as *Danaus* larvae, and the lineage of each *Danaus* replicate was recorded. Sampled adults were flash frozen in liquid nitrogen at each time point and then stored at −80 °C. Greenhouse temperatures differed only slightly between the sampling days for all larval and adult treatments: across four 24-hour collection periods, the average daytime temperature was 26.9 °C (±0.5 °C), and the average nighttime temperature was 23.0 °C (±0.5 °C) (Suppl. Table S2).

Antennae were removed from adult heads before RNA was extracted and treated as above, with the addition of an extended 15-minute centrifugation step to remove contaminating eye pigments, which were dissolved by DNase buffer. Reverse transcription and RT-qPCR were conducted as described above. *Heliothis* adult RNA was not diluted before reverse transcription, as the pigment removal treatment reduced RNA concentrations to 15 to 30 ng/µL. All RNA and cDNA samples included in our data set met the following criteria: 1) RNA concentrations above 15 ng/uL, 2) normal nanodrop readings, 3) visible band on an agarose gel, and 4) normal amplification by RT-qPCR, that is, producing only 1 melting temperature peak.

As in the larval treatments, we calculated gene expression as the ratio of target gene to reference gene molecules, which we scaled to the maximum expression value within each treatment. This scaled value controls for the differential expression of our *Heliothis* and *Danaus* reference genes, which were higher in larvae than in adults of the same species. Reference genes were stably expressed over time within each treatment (Suppl. Fig. S1). As above, we used Kruskal-Wallis testing to compare expression within and between whole time series, and we used cosinor modeling to fit a cosine curve to each series.

Comparing life history stages and species. We relied on parameter estimates from our cosinor models to compare gene expression time series between *Danaus* larvae, *Heliothis* larvae, *Danaus* adults, and *Heliothis* adults. First, we compared the percentage increase in expression from trough to peak between treatments for each gene, which we calculated as $percent\,change = \left[\left(\frac{2A+M}{M}\right)-1\right]*100\%$, where A is equal to the amplitude and M is equal to the mesor. We also compared the ratio of *per* to *tim* expression levels between treatments, which we calculated as the within-treatment difference between *per* and *tim* mesor values.

RESULTS

Larval Feeding and Activity

Feeding rhythm of Danaus larvae. When *Danaus* caterpillars fed in the temperature-controlled climate chamber, where the temperature was a constant 25 °C, 1-sample *t* tests indicated that *Danaus* larvae fed at a constant rate over the 24-hour trial, and deviation in the feeding rate was not different between time points (Kruskal-Wallis test: $\chi^2_5 = 6.3194$, $p = 0.28$). However, when *Danaus* caterpillars were moved to the greenhouse, where temperatures dropped during the nighttime, feeding was significantly higher than expected in the late photophase at ZT 13 (1-sample *t* test: $t_7 = 5.0063$, $p = 0.0016$) and ZT 16.5 ($t_7 = 6.5491$, $p = 0.0003$), and deviation in feeding was different between time points ($\chi^2_5 = 29.782$, $p < 0.0001$) (Fig. 1A).

Feeding rhythm of Heliothis larvae. When *Heliothis* caterpillars fed on an artificial diet in the temperature-controlled climate chamber (a constant 25 °C throughout day and night), feeding did not deviate from zero and was not different between time points

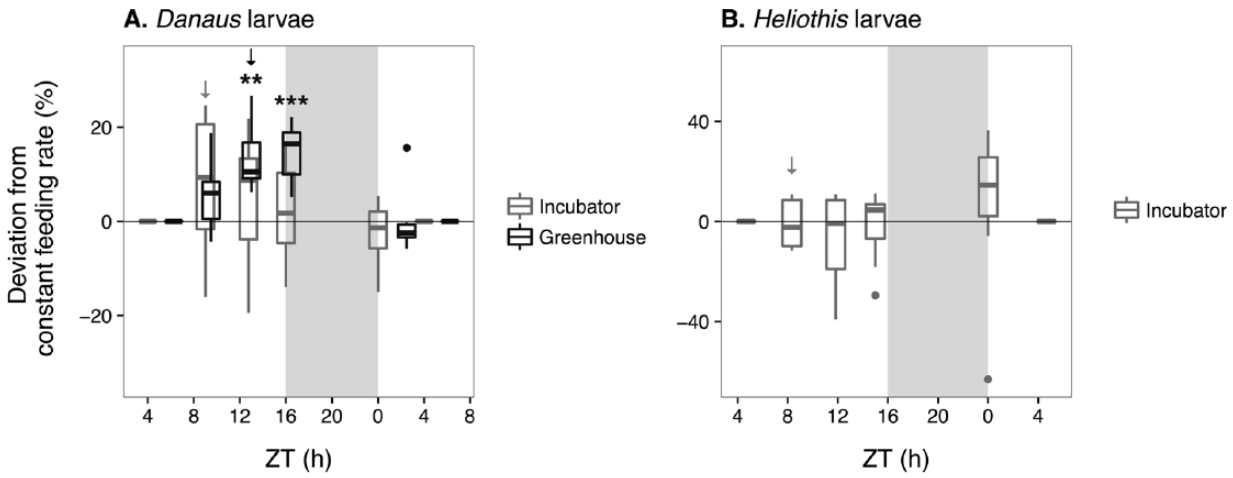

Figure 1. (A) Deviation in the constant feeding rate in *Danaus* caterpillars in the temperature-controlled incubator (gray) and in the greenhouse (black), where temperatures were colder at night. (B) Deviation in the constant feeding rate in *Heliothis* caterpillars in the incubator (gray). Boxplots represent deviation in the constant feeding rate determined for each data collection time point, represented in zeitgeber time (ZT). Arrows indicate the start of each 24-hour experiment.

(χ^2_5 = 4.2632, p = 0.52) (Fig. 1B), which indicates that *Heliothis* caterpillars also feed without a daily rhythm in constant temperature and on an artificial diet. In the greenhouse, where temperatures were lower at night, overall feeding differed between the 4 *Heliothis* caterpillar treatments (ANOVA: F_3 = 8.23, p < 0.0001); specifically, caterpillars in April on *N. benthamiana* fed more often than April caterpillars on *Z. mays* (p < 0.0001) and December caterpillars on *N. attenuata* (p = 0.004) (Fig. 2C, D). GEE models with an added treatment term better explained the relationship between feeding versus time (χ^2_3 = 12.6, p = 0.0057) and feeding versus phase (χ^2_3 = 12.6, p = 0.0056). However, phase, ZT, and treatment were not significant predictors of feeding. Importantly, in each of the 4 treatments, there was no statistical difference in feeding between day and night (GEE: December *N. attenuata*: Wald = 2.27, p = 0.13; December diet: Wald = 2.81, p = 0.093; April *N. benthamiana*: Wald = 0.71, p = 0.4; April *Z. mays*: Wald = 2.81, p = 0.094) (Fig. 2E).

To examine any potential 24-hour feeding rhythms that occur outside the binary day/night dichotomy, we attempted to fit a cosine curve to each 24-hour time series via cosinor modeling and found that none of the treatments were a significant fit, except for caterpillars feeding on *Z. mays* in April (amplitude: p = 0.0066; acrophase: p = 0.0001). Feeding in these *Z. mays* caterpillars peaked in the late scotophase at ZT 22.5 (95% confidence interval = ZT 19.75 - ZT 1.26) (Fig. 2D).

Activity rhythm of Heliothis larvae. Daily feeding patterns in *Heliothis* were different from their activity patterns, which include any amount of movements such as feeding, walking, and searching. Overall, a higher proportion of caterpillars were active compared to feeding (2-sample t test: t_{300} = −10, p < 0.0001). There were also overall differences in activity between the treatments; specifically, a higher proportion of December caterpillars on *N. attenuata* were active compared to April caterpillars on *N. benthamiana* (ANOVA: F_3 = 2.77, p = 0.044). However, the model fit of activity as a response to ZT or phase was not improved by adding a treatment term, and the treatment term was not significant in either full model (GEE: ZT: χ^2_3 = Wald = 4.98, p = 0.17; phase: χ^2_3 = 4.44, p = 0.22) (Fig. 2F, G).

In contrast to our feeding observational results, we found that in both December treatments, when there was an 8.2 °C drop in average temperature at night, caterpillars were less active at night than during the day (GEE: December *N. attenuata*: Wald = 9.11, p = 0.0025; December diet: Wald = 4.04, p = 0.044). However, in both April treatments, when the nightly drop in temperature was only 3.4 °C, caterpillar activity did not differ between day and night (GEE: April *N. benthamiana*: Wald = 0.28, p = 0.6; April *Z. mays*: Wald = 0.23, p = 0.63) (Fig. 2H).

Using cosinor modeling, we found that none of the activity data series fluctuated sinusoidally, except for the caterpillars on *Z. mays* from April (amplitude: p = 0.0008; acrophase: p = 0.0078), which was the same pattern observed for feeding. Interestingly, the daily oscillation in activity was different from their pattern of feeding, as activity peaked midday at ZT 7.04 (95% confidence interval = 4.80-9.28) (Fig. 2G).

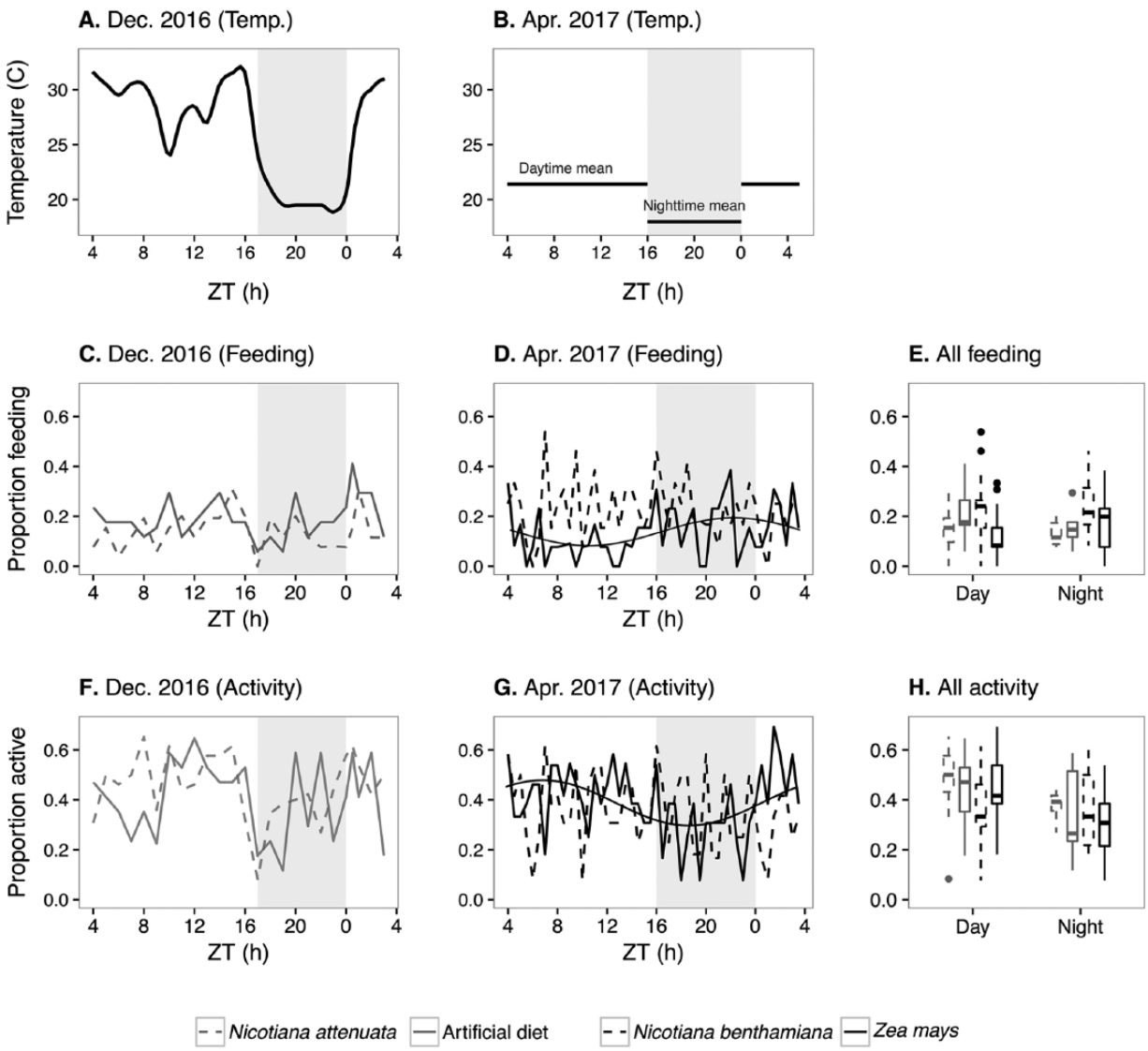

Figure 2. Two experiments with *Heliothis* larvae in the greenhouse, with varying temperatures (A, B), monitoring feeding (C-E) and activity (F-H). The proportion of caterpillars per time point feeding (C, D) and active (F, G) during the December 2016 experiment (C, F) and April 2017 experiment (D, G). Behavior was monitored on 4 host plant types: *Nicotiana attenuata* (gray dashed), artificial diet (gray solid), *Nicotiana benthamiana* (black dashed), and *Zea mays* (black solid). Black solid cosine curves show the significant cosinor model fit to *Z. mays* feeding (D) and activity (G) time series. Boxplots show the proportion of feeding (E) and active (H) caterpillars during the day and night on each host plant.

Clock Gene Expression in Larvae and Adults

Gene expression patterns in larvae. In *Danaus* larvae, transcript levels of *per* and *tim* oscillated over 24 hours (Kruskal-Wallis test: *per*: $\chi^2_{12} = 25.441$, $p = 0.013$; *tim*: $\chi^2_{12} = 22.431$, $p = 0.033$), and *per* was more highly expressed than *tim* ($\chi^2_1 = 31.263$, $p < 0.0001$) (Fig. 3A, B). In *Heliothis* larvae, rank-sum expression values were not different between time points due to high variability (*per*: $\chi^2_{11} = 10.871$, $p = 0.45$; *tim*: $\chi^2_{11} = 16.297$, $p = 0.13$); however, cosinor model parameters for amplitude (height from mesor to peak expression) and acrophase (time of peak expression) were significant for both *per* and *tim* time series, indicating that expression values oscillated sinusoidally over 24 hours. In contrast to *Danaus* larvae, *tim* expression was higher than *per* expression in *Heliothis* caterpillars (Kruskal-Wallis test: $\chi^2_1 = 29.44$, $p < 0.0001$) (Fig. 3C, D).

Comparing Heliothis caterpillars on a plant diet versus artificial diet. In both plant-reared and diet-reared *Heliothis* larvae, *per* expression was lower than *tim* expression (Kruskal-Wallis test: $\chi^2_1 = 39.503$, $p < 0.0001$). As in plant-reared larvae, diet-reared larvae also showed considerable variability in expression

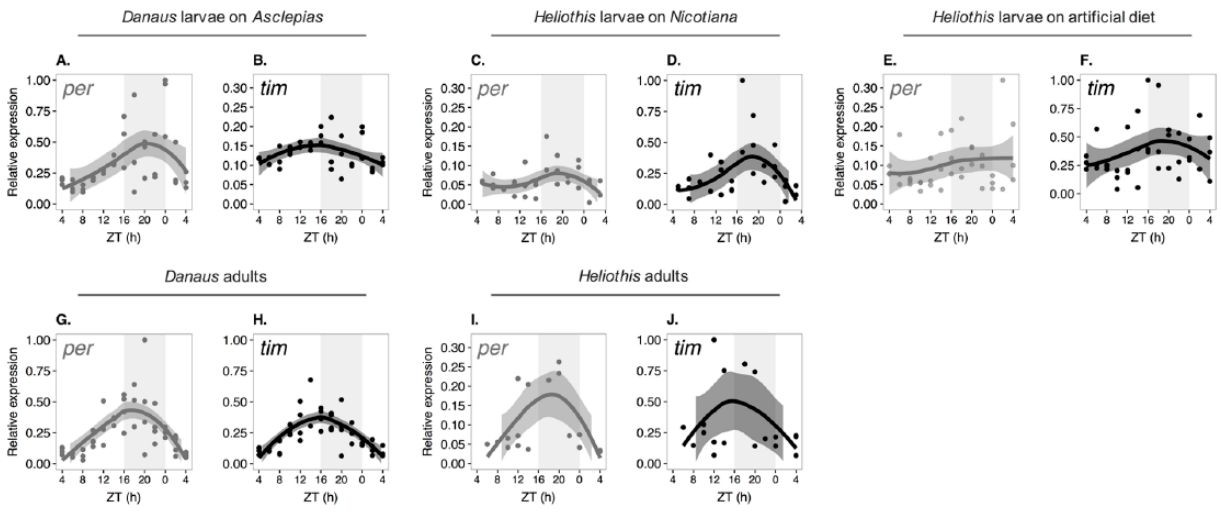

Figure 3. Relative expression of clock genes *per* (gray) and *tim* (black) over 24 hours beginning at zeitgeber time (ZT) 4 in *Danaus* larvae feeding on *Asclepias* (A, B), *Heliothis* larvae feeding on *Nicotiana* (C, D), *Heliothis* larvae feeding on an artificial diet (E, F), *Danaus* adults (A, B), and *Heliothis* adults (C, D). Gray shading indicates the scotophase. Trend lines were fitted using local regression with 95% confidence intervals.

values, particularly in the *tim* gene, such that rank-sum expression values were not different between time points (diet *per*: $\chi^2_{12} = 13.595$, $p = 0.33$; diet *tim*: $\chi^2_{12} = 11.516$, $p = 0.49$) (Fig. 3E, F).

However, *per* and *tim* expression was statistically different between plant-reared and diet-reared *Heliothis* larvae, both in the amount of mRNA expressed as well as the shape of the curve itself. Overall expression levels for both *per* and *tim* were higher in diet-reared larvae than in plant-reared larvae (Kruskal-Wallis test: *per*: $\chi^2_1 = 7.3167$, $p = 0.0068$; *tim*: $\chi^2_1 = 7.2835$, $p = 0.007$). Additionally, even though cosinor model parameters were significant for both the *per* and *tim* time series in plant-reared larvae, neither amplitude nor acrophase was significant in diet-reared larvae, indicating a nonsignificant model fit.

Gene expression patterns in adults. In *Danaus* adults, expression levels of *per* and *tim* also oscillated over 24 hours (Kruskal-Wallis test: *per*: $\chi^2_{12} = 36.402$, $p = 0.0003$; *tim*: $\chi^2_{12} = 34.183$, $p = 0.0006$), and levels of *tim* and *per* expression were not different from each other ($\chi^2_1 = 0.032$, $p = 0.86$) (Fig. 3G, H). Expression levels were also not different between male and female adults (*per*: $\chi^2_1 = 2.3533$, $p = 0.125$; *tim*: $\chi^2_1 = 1.7952$, $p = 0.18$) or between lineages (*per*: $\chi^2_3 = 1.2571$, $p = 0.7393$; *tim*: $\chi^2_3 = 1.2185$, $p = 0.75$) (data not shown). *Heliothis* adult *per* and *tim* expression time series were less robust than the other treatments, which is most likely due to the fact that the harsh pigment removal treatment rendered many replicates unsuitable for RT-qPCR; thus, rank-sum expression values were not different between time points (*per*: $\chi^2_9 = 12.327$, $p = 0.2$; *tim*: $\chi^2_9 = 5.3801$, $p = 0.83$). However, despite the large standard error and low sample size, the shape of the expression curves was similar to that of *Heliothis* larvae. As in *Heliothis* larvae, *per* expression was lower than *tim* expression in *Heliothis* adults (Kruskal-Wallis test: $\chi^2_1 = 11.036$, $p = 0.00089$) (Fig. 3I, J). Expression levels were not different between male and female adults (*per*: $\chi^2_1 = 2.3882$, $p = 0.1223$; *tim*: $\chi^2_1 = 2.8421$, $p = 0.092$) (data not shown).

Comparing life history stages and species. All expression time series had overlapping confidence intervals for the acrophase parameter according to the cosinor model, indicating that *per* and *tim* were expressed with a similar phase across larvae and adults of both species (see Suppl. Table S3 for full cosinor parameter values). The fold change in expression from trough to peak was lower in *Danaus* larval *tim* than in *Danaus* adult *per*, adult *tim*, and larval *per*. In *Heliothis*, there was no difference in fold change between larvae and adults reared on a diet or on plants for either gene (Suppl. Fig. S2). Interestingly, there were interstage and interspecific differences in the relative expression of *per* and *tim* within the brain. Both *Heliothis* larvae and adults expressed *per* about 200% to 300% more highly than *tim*, whereas *Danaus* adults expressed *per* and *tim* in equal quantities, and *Danaus* larvae expressed *tim* more highly than *per* (Suppl. Fig. S3).

DISCUSSION

Whereas activity rhythms in adult butterflies are limited almost exclusively to the photophase and activity in moths is limited almost exclusively to the scotophase (Ramaswamy, 1990; Brower, 1995; Brower, 1996; Groot, 2014), our caterpillars fed throughout the day and night. However, we found that both larvae and adults possess the underlying machinery required for the ultimate regulation of circadian rhythms—that is, cycling expression of their core clock genes *per* and *tim*. Our comparative study of clock gene expression between larvae and adults of 2 lepidopteran species further uncovered expression differences between genes (*per* v. *tim*), life stages (particularly *Danaus* larvae v. adults), and species (butterfly v. moth) that reveal surprising differences in the regulation of clock gene expression, which may cause differences in clock function itself between lepidopteran life stages or species.

Our results suggest that environmental variables such as temperature and host plant may drive slight fluctuations in feeding and/or activity in the larval stage. We found that under constant temperature, feeding rhythms in both larval species were not present; however, a cooler nighttime temperature caused a nighttime reduction in *Danaus* feeding and *Heliothis* activity but not in *Heliothis* feeding. In *Heliothis* larvae in particular, our behavioral observations showed that a temperature fluctuation of 8.2 °C from day to night was sufficient to cause daily differences in activity but not feeding; however, a fluctuation of only 3.4 °C did not stimulate a day-night rhythm in either activity or feeding. Both activity and feeding in *Heliothis* did, however, oscillate sinusoidally over 24 hours when the caterpillars fed on corn plants under fluctuating daily temperatures.

It is unclear whether temperature and host plant cause behavioral rhythms through entrainment to the endogenous larval clock or are unconnected to clock processes. Temperature entrainment does not appear to be required for stimulating daily feeding rhythms in other caterpillar species, as *Trichoplusia ni, Spodoptera exigua*, and *Spodoptera littoralis* caterpillars exhibit feeding rhythms in constant temperature (Goodspeed et al., 2012; Kim and Hong, 2015; Suszczynska et al., 2017). The feeding rhythms of at least 2 of these species, *T. ni* and *S. littoralis*, persist in constant darkness, suggesting circadian regulation. The extent to which circadian-governed behavior occurs in the approximately 165,000 species of caterpillars, however, remains an open question. A handful of other caterpillar species do exhibit rhythmic daily changes in feeding and activity within their native habitats, which may or may not be under endogenous control. Caterpillars that have been observed feeding primarily at night include *Morpho peleides limpida* (butterfly), *Lymantria dispar* (moth), and late instars of *Malacosoma americanum* (moth) (Leonard, 1970; Young, 1972; Fitzgerald et al., 1988). Some species, such as *L. dispar*, remain inactive during the day in shady locations to prevent overheating (Casey, 1976), while other species, such as *M. americanum*, probably feed at night as a strategy of predator and parasitoid avoidance (Fitzgerald et al., 1988), particularly in later instars that would be more susceptible to detection (Reavey, 1993). However, a number of species have also been noted as daytime feeders, including *Chlosyne lacinia* (butterfly) and *Malacosoma disstria* (moth) (Stamp, 1977; Mcclure and Despland, 2010).

In several caterpillar species (e.g., *Pieris rapae, Hyles lineata*, and *Manduca sexta*), feeding and activity rates are tightly coupled with body temperature, which is in turn influenced by air temperature (Casey, 1976; Kingsolver and Woods, 1997; Kingsolver, 2000). In *M. sexta*, for example, as the air temperature increases, the consumption rate rises in tandem (Reynolds and Nottingham, 1985), while under constant air temperature, caterpillars feed at a constant rate throughout the day and night (Casey, 1976; Herden et al., 2016). We suspect that a similar causative relationship between temperature and behavior may underlie the slight diurnal patterns that we produced in our 2 caterpillars. Although the daily temperature fluctuation was mild in our experiment (~8 °C), *Heliothis* caterpillars typically endure daily fluctuating temperatures of 15 °C or more in their native range (Fitt, 1989); this larger disparity between daytime and nighttime temperatures could well cause increasingly disparate daytime and nighttime behavior outside the laboratory.

We also tested the role of host plant as a potential manipulator of daily *Heliothis* behavior and found that both feeding and activity oscillated sinusoidally over 24 hours on only one plant: the corn plant (*Z. mays*). Intriguingly, while activity on *Z. mays* peaked midday, feeding actually peaked towards the end of the night, suggesting that diurnal *Z. mays* defenses may suppress daytime feeding. Daily fluctuating plant compounds involved in direct and indirect defense have been shown to interrupt daytime feeding and drive nocturnal feeding patterns in other caterpillar species. Diurnal peaks of the direct defensive compound jasmonic acid in *Arabidopsis thaliana* constrain daytime feeding in the caterpillar *T. ni* (Goodspeed et al., 2012), while *Mythimna separata* caterpillars use diurnal cues from indirect defensive compounds, herbivore-induced plant volatiles (HIPVs), to regulate their behavior and feed more at night on *Z. mays*, irrespective of the light condition (Shiojiri et al., 2006). However, the effect of diurnal

plant defenses on caterpillar behavior may rely on the interaction between plant and caterpillar species: *Nicotiana* defenses do not suppress daytime feeding in either our *Heliothis* caterpillars or *M. sexta* caterpillars (Herden et al., 2016), and 2 other moth species (*Mythimna unipuncta* and *Spodoptera litura*) do not respond directly to diurnal *Z. mays* HIPVs (Shiojiri et al., 2011). Future studies could build upon our results by specifically examining how the diurnally oscillating *Z. mays* lipoxygenase *ZmLOX10* and other *LOX10*-dependent direct and indirect defensive compounds, including both jasmonic acid and HIPVs, affect daily caterpillar behavior on corn plants in particular (Nemchenko et al., 2006; Christensen et al., 2013).

Although feeding and activity in *Danaus* and *Heliothis* caterpillars may or may not be affected by the molecular clock, we found that caterpillars do possess the cycling clock gene expression characteristic of an intact circadian oscillator. The larval oscillator is likely used to coordinate other nonbehavioral circadian rhythms, including rhythms in development and endocrine activity (Truman, 1972; Saunders, 2002), although other endogenous behaviors missed by our data collection methods may also exist (e.g., the duration of feeding bouts) (Bernays and Singer, 1998; Nagata and Nagasawa, 2006). Our results are aligned with previous indications that clock genes generally cycle in nymphs and larvae. *per* and *cyc* mRNA oscillates in the nymphal brains of the linden bug *Pyrrhocoris apterus*, which likely functions as a photoperiodic timer (Kotwica-Rolinska et al., 2017). In caterpillar larvae, PER was first stained in the *A. pernyi* brain 20 years ago (Sauman and Reppert, 1996), and *per* mRNA has been recently found to cycle in the fat body of *S. littoralis*, although it is arrhythmic in the gut (Suszczynska et al., 2017).

We also hypothesized that plant circadian rhythms (in secondary metabolite and volatile production, for instance) could act as zeitgebers to the larval circadian clock, and we found that clock gene expression indeed differed between plant-reared and diet-reared *Heliothis* larvae; transcript levels were slightly higher in the diet-reared larvae, and notably, sinusoidal gene expression oscillation was less robust when caterpillars fed on a diet. The rhythmic cycling of clock gene mRNA is a fundamental property of circadian clocks and a necessary mechanism for accurate time keeping and ultimate regulation of rhythms. When the cyclical expression of *per* and *tim* is disrupted, endogenous behavioral rhythms are often also disrupted (Bell-Pederson et al., 2005). These results indicate that feeding substrate could potentially alter core functioning of the insect clock by reducing the robustness of clock gene cycling. Although we did not find major differences in daily feeding or activity between *Heliothis* caterpillars on *N. attenuata* plants versus an artificial diet in our observational experiments, it is possible that feeding substrate–based modification of clock gene expression could affect established downstream circadian rhythms in larvae, but future studies will further explore the validity of this novel hypothesis.

Some aspects of clock gene expression were similar between life history stages and species. Importantly, the expression of *per* and *tim* in both larvae and adults of *Danaus* and *Heliothis* oscillated with a similar phase. Phase angle (i.e., the time of peak expression relative to the light-dark cycle) is an essential functional property of the clock to which downstream rhythms synchronize (Herzog, 2007). Our *Danaus* and *Heliothis* time series also contained a similar phase angle to several other lepidopterans including *A. pernyi, B. mori*, and *E. kuehniella* (Reppert et al., 1994; Iwai et al., 2006; Kobelkova et al., 2015), with peak expression occurring in the early to middle scotophase. However, the expression of *per* in the fall armyworm *S. frugiperda* has been shown to peak late, between 6 and 10 hours into the scotophase (Hänniger et al., 2017), indicating that the phase angle may not be completely conserved between all lepidopteran clocks. Another similarity between our treatments was expression fold change over 24 hours, which overlapped not only in adults of both species but also with previously quantified mRNA fold change values reported for *D. plexippus, B. mori*, and *A. pernyi* adults (Reppert et al., 1994; Iwai et al., 2006; Zhu et al., 2008).

Despite these similarities, we found differences in clock gene oscillation between *Danaus* life stages and between species. In both *Heliothis* larvae and adults, *per* was more highly expressed than *tim*, whereas *Danaus* adults expressed *per* and *tim* in equal quantities, and *Danaus* larvae expressed *tim* more highly than *per*. Additionally, *Danaus* larval *tim* had a lower expression amplitude than other *Danaus* treatments. It is unclear what drives these differences or how this is related to the circadian phenotype. Temperature, photoperiod, and light intensity have all been suggested to affect *per* and *tim* expression amplitude and cycling (Pittendrigh et al., 1991; Iwai et al., 2006; Montelli et al., 2015); however, as our species were reared in the same environmental conditions, this explanation alone is not sufficient to explain differences in the *per:tim* ratio as well as the dampened amplitude of *Danaus* larval *tim*.

Reconstruction of the butterfly brain during metamorphosis may play a role in prescribing the differences in clock gene expression between *Danaus* life stages. During butterfly metamorphosis, new neurons are formed, and some old neurons are remodeled

(Truman, 1972). In *Rhodnius prolixus* (order Hemiptera), new clock cells differentiate, and circadian circuitry increases in complexity during metamorphosis (Vafopoulou and Steel, 2012). In *Danaus*, the rearrangement and creation of clock neurons during metamorphosis could potentially account for differences in clock gene expression between life stages. However, we did not see a disparity in clock gene mRNA levels or cycling amplitude between stages in *Heliothis*, suggesting that the metamorphic reorganization of clock components could be species specific.

Ultimately, insect oscillators are immensely complex, and *per* and *tim* expression could be shaped by any number of interactions from pretranscription to posttranslation. For example, interspecific differences in the *per:tim* expression ratio or in *Danaus* larval *tim* could involve differences in CLK-CYC binding rhythms or posttranscriptional regulation, which have been shown to affect *per* and *tim* expression (Taylor and Hardin, 2008; Lim and Allada, 2013; Rodriguez et al., 2013). Alternatively, differences in mRNA stability could affect amplitude and cycling in the absence of posttranscriptional correction, or lowly expressed genes may cycle in fewer areas of the brain (Abruzzi et al., 2011; Lim and Allada, 2013). Similarly, anatomic differences in optic lobes and compound eyes may cause expression differences between stages. Dissimilarity in expression between treatments might also arise from divergent clock responses to temperature or other environmental variables; for instance, *per* expression levels in *Drosophila melanogaster* are low in warm temperatures, while the opposite is true for *tim* expression (Majercak et al., 1999; Maguire et al., 2014; Montelli et al., 2015). Further testing is needed to uncover the mechanisms that bring about differences in *per* and *tim* expression between life stages and species.

Other studies have also revealed that different clock gene expression levels, even in the absence of phase shifts, can be associated with differential circadian activity. Clock gene expression levels differ in the passerine bird *Emberiza melanocephala* between their diurnal premigratory life history stage and their nocturnal migratory stage (Singh et al., 2015). Additionally, *per* and *tim* are expressed more highly in the diurnal adults of the cricket *Gryllus bimaculatus* than in the nocturnal nymphs (Uryu and Tomioka, 2014). Our study shows that similar core clock expression divergences also exist between lepidopteran species and even between life stages of the same butterfly species; however, it remains unclear if or how these differences affect circadian behavior.

As a whole, our study has defined the similarities and differences between life stages and species in both rhythmic behavior and gene oscillation. While butterfly and moth adults have oppositely timed circadian behavioral rhythms, behavior in their larval stage is likely not clock driven but rather shaped by environmental factors. Clock gene cycling across all treatments, however, shows that the circadian oscillator functions as a timekeeper in both larvae and adults, and the conservation of phase angle across treatments shows that opposite behavioral rhythms in the adult stage do not arise from shifting phase angles of *per* and *tim* expression. However, absolute expression differences between *per* and *tim* as well as slight differences in the shape of time series between treatments indicate that the oscillator is not mechanistically identical between caterpillars reared on live plants versus a diet, between life history stages, or between species. Further investigation will clarify how these expression differences arise and whether they have a functional downstream effect on resulting circadian rhythms.

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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NOTE

Supplementary material for this article is available online.

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