Article

Adult diel locomotor behaviour in the agricultural pest *Plutella xylostella* reflects temperature-driven and light-repressed regulation rather than coupling to circadian clock gene rhythms.

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**Simple Summary:** Insect pests such as the diamondback moth, which infests cabbages and related crops, inflict massive amounts of damage to agriculture that costs billions of US$ annually. Since the adult diamondback moths spread by migrating, factors controlling their activity and movement are of interest. Daily and seasonal activity rhythms in many animals are regulated by innate daily timekeeping systems termed circadian clocks. In this study we describe rhythms in the molecular elements of this timekeeping system in diamondback moth and we test if the circadian clock can be held responsible for daily rhythms in moth activity. We found that diamondback moth activity was suppressed by light and regulated by environmental temperature rather than the circadian clock. These insights are helpful in predicting the activity of this agricultural pest.

**Abstract:** The diamondback moth, *Plutella xylostella,* is arguably the most economically impactful and widespread lepidopteran pest. Though the larval *P. xylostella* life stage is responsible for most of this cost through the consumption of crops, it is the adult form that spreads the pest to fresh crops all around the world, seeking them out in a seasonally expanding range. It is therefore important to understand the activity rhythms of adult *P. xylostella* in response to environmental cues such as light and temperature. We analysed diel rhythms in both adult clock gene expression and locomotor behaviour for the ROTH *P. xylostella* strain. Real-time quantitative PCR analyses of *P. xylostella* demonstrated diel rhythms for transcripts of the clock genes *period* and *timeless* under both entrained and free-running conditions indicating the presence of a functional daily timekeeping mechanism. However, adult locomotor rhythms exhibited temperature-driven and light-repressed regulation rather than circadian control. Thus, our analyses show a lack of coupling between the *P. xylostella* circadian clock and adult locomotor behaviour, which may be relevant in predicting the activity patterns of this agricultural pest.

**Keywords:** diamondback moth; behavioural rhythm, daily timekeeping, light response; temperature response

1. Introduction

Climate change and an exponentially growing human population are putting pressure on arable land, threatening global famines before the end of the century [1], making food security a priority issue that needs to be addressed [2]. A key factor in the challenge to maintain food production is the control of pest species, with the effects of pests underestimated [3] and increasing in severity as the climate changes [4-7]. *P. xylostella* (diamondback moth) is a pest species that has taken hold globally, having achieved a cosmopolitan distribution with a seasonally expanding range that covers all but the coldest latitudes [8,9]. *P. xylostella* is a *Brassicaceae* specialist herbivore [10] causing worldwide losses estimated at ~$5 billion annually [11], up from ~$1 billion in the 1990’s, making *P. xylostella* arguably the world’s most costly lepidopteran pest [8]. *P. xylostella* challenges farmers due to rapid proliferation in newly colonized fields, able to produce 250+ eggs per female [12] and complete multiple life cycles per season [8,13]. *P. xylostella* also shows rapid pesticide resistance gain [14-16], leading to control failures with some regions facing >90% crop losses [17,18]. Integrated Pest Management (IPM) strategies are being implemented to both diminish pesticide use and *P. xylostella’s* impact [19-21], however, the uptake of these strategies has been limited, with the prohibitive complexity of treatments needed for success causing farmers to fall back onto aggressive pesticide spraying protocols [22-24]. The study of behavioural manipulation as part of control strategies has been gaining increasing attention as these research pathways may compliment and help increase the efficacy of IPM [25-27].

The circadian clock acts as an endogenous time keeping mechanism controlling processes in organisms to best fit the daily rhythms of the planet [28,29]. This clock runs via a series of transcriptional and translational feedback loops (TTFL) that maintain ~24h rhythms without the need for external stimuli such as light and temperature though it can be entrained by such. The lepidopteran circadian clock differs from the *D. melanogaster* model organism for arthropods [30,31], having two CRYPTOCHROME (CRY) proteins active in the TTFL. CRY2 (vertebrate-like CRY) acts as a negative regulator, in the complex that represses *per, tim* and *cry2* expression [32,33].

Circadian rhythms have a widespread impact on gene expression. For example, ~40% of coding genes were found to exhibit circadian rhythms in mice [34] and ~35% in plants [35]. Synchrony of internal and environmental rhythms is important for health and well-being with circadian clocks exerting a strong influence over organisms’ immune systems and anticipation of threats [36-39]. Lepidopteran circadian mechanisms have been shown to influence numerous behaviours including life stage cycles, oviposition [40] and seasonal migrations [41]. *P. xylostella* may better adapt to local environments and rhythmic changes via seasonal migration. In other species, seasonal migration is known to be informed by clock-dependent detection of relevant changes in photoperiod [42,43] and temperature [44,45]. Moreover, the way in which *P. xylostella* controls and modulates its behaviour when invading new regions and after invasions have taken place may be important for informing control measures as well as complimenting and improving the efficacy of IPM strategies. *,* which lack an ability to diapause, with populations sustained [9,46,47] Modulating photoperiod in a neotropical *P. xylostella* population had no discernible effect morphology or life history [48]. However, systematic analyses of the impact on *P. xylostella* physiology and behaviour of changes in both photoperiod and daily temperature cycles remain to be conducted.

It is therefore important to understand both *P. xylostella*’s daily time keeping mechanisms and the rhythmic behavioural outputs that occur in association with these. Molecular circadian rhythms underlying daily timekeeping were explored by determining transcript profiles of the *P. xylostella* clock genes *per* and *tim*. In addition, *P. xylostella* were studied for their diel locomotor activity under a range of environmental light and temperature cycles. Our behavioural analyses of the ROTH *P. xylostella* strain maintained on *Brassica rapa* complement a prior behavioural study using the Geneva 88 *P. xylostella* strain maintained on artificial diet that identified nocturnal behaviour with relatively weak male-specific circadian behaviour [49].

2. Materials and Methods

2.1. P. xylostella culture maintenance

*P. xylostella* populations were reared within an environmental control room (ECR) at 20°C in a 12/12 hours Light/Dark (L/D) light cycle. W30xD30xH30cm BugDorms were used to house mixed sex populations (BugDorm, 2019). The population was acquired from Rothamsted Research, (ROTH strain wild collections during the 1960s) and has been continuously maintained on *Brassica rapa*. A sugar-water source was provided for adults during egg laying period with adults mixed between new cages to prevent genetic isolation.

2.2. P. xylostella locomotor activity assays

In preliminary activity monitor experiments, three different substrates were trialled. Cotton wool saturated with sugar-water or diluted honey versus sugar-agar solid media. As moths often got stuck and/or wet when sugar/honey solution-saturated cotton wool was used the solid sugar-agar media were used instead. To control fungal growth this media was prepared with 0.07% Methyl 4-hydroxybenzoate. This was added using a 10% Methyl 4-hydroxybenzoate w/v stock solution in ethanol after 1 % agar 5 % table sugar media had been boiled and cooled down to ~60°C. During preparation 25 mm diameter x 95 mm length glass test tubes were filled from the bottom with 2 cm with the media. Media was left to dry before adding individual *P. xylostella* under CO2 anaesthesia and the tubes were capped with a cellulose acetate to allow gas exchange. Individual *P. xylostella* activity counts were detected with the tubes in horizontal orientation by TriKinetics LAM25 assay system using (TriKinetics, 2019a,b). Based on increased mortality at 23°C in preliminary experiments, assays were conducted at 17°C or 20°C.

For individual locomotor assays at constant temperature LAM25 monitors were kept inside a black plastic box at 17°C with local humidity provided by a tray of water containing algicide and biocide to suppress microbial growth. White LEDs were mounted inside the black plastic tub providing 8µmol/m2/s (~400 lux) light at 12/12, 6/6/6/6, 14/10, 16/8, 18/6 and 20/4 hours of light (L) and dark (D) cycles or at constant light (L/L) or constant darkness (D/D), shown in **Table 1**. In parallel, environmental cycles mimicking light and temperature recordings in Kent, UK in April and June, were set up for individual locomotor activity assays in an Percival incubator, model number: DR-36VL, (Clf Plantclimatics, 2019) based on environmental data from Shaw et al. 2019, producing the conditions shown in **Table 1**. The recorded environmental profile in the incubator setups is shown in **Suppl. Fig. S4**, where a programmable LED lamp (FLUVAL14521) was used to create ramping dawn and dusk light with the incubator maintaining the temperature profile, with max light producing 38µmol/m2/s (~2000 lux). Gradually ramping temperature cycles in (D/D) and (L/L) conditions were produced in the same incubators, using the four built-in fluorescent lights to provide the light for the latter condition. For each locomotor assay, *P. xylostella* were selected freshly from culturing enclosures and were recorded over 6+ days. The data was collected by a DAM (Drosophila Activity Monitor) system [50] in 5 min segments and was subsequently exported to ClockLab Analysis Version 6 [51] for analysis. DEnM monitors were used to keep track of experimental light, temperature and humidity conditions.

**Table 1.** Diel environmental conditions for locomotor activity assays. Dawn refers to the phase of increasing light intensity, dusk refers to phase of decreasing light intensity. .

|  |  |  |
| --- | --- | --- |
| **Condition** | **Light cycle** | **Temperature** |
| D/D | Constant Dark | 17°C |
| D/D | Constant Dark | 20°C |
| 12/12 | 12h Light, 12h Dark | 17°C |
| 6/6/6/6 | 6h Light, 6h Dark, 6h Light, 6h Dark | 17°C |
| 14/10 | 14h Light, 10h Dark | 17°C |
| 16/8 | 16h Light, 8h Dark | 17°C |
| 18/6 | 18h Light, 6h Dark | 17°C |
| 20/4 | 20h Light, 4h Dark | 17°C |
| L/L | Constant Light | 17°C |
| ‘April’ | 4h Light, 5h dusk, 10h Dark, 5h Dawn | 10°C-16°C |
| ‘June’ | 15.5h Light, 1.5h dusk, 5.5h Dark, 1.5h dawn | 14°C-20°C |
| D/D temp | Constant Dark | 14°C-22°C |
| L/L temp | Constant Light | 14°C-22°C |

2.3. P. xylostella RNA collection and extraction

Whole adult *P. xylostella* male moths were maintained at 20C under 12h:12h L/D conditions and then collected every 4h during the last day of L/D and the first day of subsequent D/D conditions (ZT1-5-9-13-17-21-CT1-5-9-13-17-21). Male moths were used exclusively to avoid confounding effects due to sex ratio fluctuations or female egg load [52]. Adult males were snap frozen at -80°C for storage and used to extract RNA using RNAqueous-4PCR kit (ThermoFisher, 2021a). The isolated RNA was treated with DNase 1 and the resulting RNA samples were tested for concentration and quality using a NanoDrop 1000 spectrophotometer (ThermoFisher, 2021b). Samples were aliquoted into an appropriate working volume (used for specific Quantitative Polymerase Chain Reaction (qPCR) plate set ups) and were kept at -20°C for short term storage.

2.4. qPCR protocol

RNA samples were used as template for one-step amplification reverse transcriptase quantititave PCR (qPCR) with PrecisionPLUS OneStep qRT-PCR Master Mix (Primerdesign, 2021) with both a SYBR green florescent dye and ROX dye. qPCR was performed using a StepOnePlus Real-Time PCR System [53] to quantify proportional levels of specific RNA transcripts between samples [54]. Amplification thresholds were used to calculate the proportional level of primer-specific transcripts present in each RNA sample using the 2-ΔΔCT method [55]. *Elongation factor 1 α* (*Ef1α*) was used as a housekeeping reference gene in order to analyse the relative expression of circadian genes *period* (*per*) and *timeless* (*tim*) shown in **Table 2**. Primers were designed using Primer-3 and Primer-BLAST [56,57] to amplify desired distinct gene transcripts. Adherence to the MIQE guidelines was submitted along with the research [58].

**Table 2.** Primer pair sequences and amplicon lengths for *P. xylostella* genes of interest used in qPCR experiments, adapted from [59] and tested using qPCR serial dilution for efficiencies and 5% agarose gel electrophoresis to check expected amplicon lengths.

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| --- | --- | --- | --- | --- |
| **Primer Pair** | **Forward sequence** | **Reverse sequence** | **Amplicon length (bp)** | **Efficiencies** |
| *Ef1α* | 5’-GCCTCCCTACAGCGAATC-3’ | 5’-CCTTGAACCAGGGCATCT-3’ | 162 | 98.7% |
| *per* | 5’-CCGCGAAAGAACGTCTAAGG-3’ | 5’-GTGCTCGTGGTCGTGGTTA-3’ | 118 | 108.7% |
| *tim* | 5’-ACGCTGCTGAGAAATGGACA-3’ | 5’-CCGCTATCAGGTCCGATGAC-3’ | 87 | 105.9% |

2.5. Statistical analyses

ClockLab Analysis Version 6 software was used to analyse and produce graphical representations of *P. xylostella* locomotor activity data at 30 min resolution in the form of double-plotted actograms, daily activity profiles and chi-square periodograms and to quantify specific associated parameters of both individual moths and populations. Individual moths were assumed to have died and were excluded from the results if their activity stopped permanently during a 6-day analysis interval. Flies were considered rhythmic if they exhibited a significant (p<=0.01) chi-square periodogram amplitude for a period in the circadian range (15-35h). Relative rhythmic power (RRP) was calculated as the ratio between that amplitude and the associated significance threshold value. Shapiro-Wilk tests were used to test if data met the assumptions required for parametric testing, e.g. normal distribution of data.

Where the assumption of normality was consistently found to be met comparisons were performed using t-tests or ANOVA with Tukey’s multiple comparisons analysis. In cases, where some of the data sets violated this assumption nonparametric analyses (Mann-Whitney U test, Kruskal-Wallis tests with Dunn’s multiple comparison post hoc tests) were used instead. Differences between light and dark phase activity counts of individual moths were assessed using Wilcoxon matched pairs signed rank test. Simple linear regression was used to describe the relationship between dark phase length and average dark phase activity counts. The resulting equation was then compared to models assuming constant daily activity or a constant activity rate during the dark phase. Two-sided permutation t-test estimation statistics [60] was used to illustrate the distribution of activity across two halves of experimental days. The CircaCompare R package [61] was used for the calculation and comparisons of rhythmic activity and expression profiles using rhythmic features (mesor, Amplitude and Phase) to compare rhythmic patterns between groups of data through cosinusoidal curve fitting.

3. Results

3.1. P. xylostella circadian clock gene rhythms

The rhythmic profiles of the clock gene transcripts for *tim* and *per* relative to a reference gene (*Ef1α*) were determined by qPCR from total RNA extracted from adult male moths across a 2-day L/D-D/D time course sampled in at 4h resolution (**Figure 1, Table 3**). Significant rhythmicity was observed for each clock gene under each environmental condition use cosinusoidal fitting (**Supplementary Figure S1**, **Supplemental Table S1**). The transcript profiles for *per* and *tim* closely matched across the entire time course with no significant difference at any time point (**Figure 1, Table 3**). Both transcripts exhibited a major increase in association with the onset of dusk or subjective dusk.

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**Figure 1.** qPCR analysis of *per* and *tim* transcript levels in adult male *P. xylostella* across a 48h L/D-D/D time course at the indicated L/D (ZT) and D/D (CT) time points. The line graphs show average±SEM. Relative expression normalised to a reference transcript (*Ef1a*). Six independent biological replicates were used.

**Table 3.** CircaCompare analysis of *per* and *tim* qPCR L/D-D/D transcript profiles. The Rhythm pvalue corresponds to the data’s cosine fit; Mesor represents a rhythm-adjusted mean; Amplitude reflects the peak-to-trough difference of the fitted curve; Phase peak refers to the daily maximum of the fitted curve.

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| --- | --- | --- | --- | --- |
| **Condition** | **Rhythm p value** | **Mesor** | **Amplitude** | **Phase peak** |
| *per* | 3.19-7 | 1.11 | 0.34 | 16.86 |
| *tim* | 3.89-7 | 1.14 | 0.42 | 17.12 |

3.2. P. xylostella individual adult locomotor rhythms at constant temperature

*P. xylostella* diel locomotor behaviour was assessed on sugar/agar substrate using Trikinetics LAM25 monitors at constant 17°C for D/D and L/L conditions as well 12:12, 14:10, 16:8, 18:6 and 20:4 L/D cycles and 6:6:6:6 L/D/L/D cycles (see **Figure 2, 3, supplemental Figure S2, Table** **4, supplemental Table S2,S3**). While locomotor behaviour was rhythmic under all L/D cycles this was not the case for constant D/D or L/L conditions. Moreover, diel activity profiles did not reveal anticipatory features. Activity was increased immediately upon transition from light to dark and then dropped off to a lower level, while the onset of light triggered an immediate and sustained drop in activity. A prior study observed that when placed in 6:6:6:6 L/D/L/D photoperiods wild-type, but not arrhythmic mutant *Drosophila melanogaster* exhibited preferential rhythmicity in the circadian range rather than the entrained 12h L/D cycle [62] such that increased locomotor activity was observed every other 6:6 L/D cycle. However, as is evident by the activity profiles and actograms (see **Figure 2, supplemental Figure S2)** as well as comparative analyses of activity levels during the first and second 6:6 L/D phases per cycle (see **Figure 3**) this was not the case for *P. xylostella*. In fact, the differences between the first and second 6:6 L/D phases were no more pronounced than those for the subjective dark versus subjective light phases of the D/D condition, where behaviour was arrhythmic. Thus, no evidence of circadian control of adult locomotor behaviour was uncovered. Instead of clock-mediated rhythmicity the observed diel locomotor activity patterns exhibited two other regulatory features: (1) light-mediated repression and (2) homeostasis. Beyond the qualitative changes in the activity profiles and actograms light-mediated repression was demonstrated quantitatively in the significantly reduced activity counts under L/L conditions (**Figure 2B, supplemental Figure S2B**), preferential activity during the dark phase of L/D cycles versus the subjective dark phases of D/D or L/L cycles (**Figure 2D, Figure 3A-C, supplemental Figure S2D**). The data also indicated homeostatic control of diel locomotor activity in the dark as total diel activity was relatively similar across L/D cycles with dark phases varying from 4 to 12h (**Figure 2B, supplemental Figure S2B**), while dark phase activity rate was lowest in D/D (**Figure 2C, supplemental Figure S2C**). Given very strong light-mediated repression, diel dark phase activity would be expected to increase proportionally with dark phase length in the absence of homeostasis. In contrast, homeostatic maintenance of a fixed diel activity level would require proportional decreases in dark phase activity rates with increasing diel dark phase length. In fact, the observed adult male and female locomotor activity data was best fit by an intermediate model exhibiting some level of homeostasis (**Figure 4**), Though light repression was strong, it was incomplete as the light phase contributed more to total activity activity in L/D cycles with short dark phases (18:6, 20:4) versus those with equal amounts of light and dark (12:12, 6:6:6:6; **Figure 2D, supplemental Figure S2D**).

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**Figure 2.** Locomotor behavioural analysis of adult male *P. xylostella* under different lighting conditions. A) Left: Normalized average ± SEM daily activity profiles over 6 day intervals for *P. xylostella* adult males. Time is plotted along x-axis in hours (h), starting at the onset of dark or subjective dark phase with normalized activity indicated by the y-axis. Right: double-plotted actograms of normalized average activity of moths over 6 days in 30 minute bins. The horizontal bars along the top of individual activity profiles and actograms indicate dark phase (black), subjective dark phase (grey cross hatching), light phase (white) and subjective light phase (grey). B) total average daily activities, C) average hourly dark phase activity, D) average % of total activity occurring during (s)dark phase, E) average % of total activity occurring during first 2 h of (s)dark phase. Data points outside Tukey range (1.5XIQR (Interquartile range)) are shown. Kruskal-Wallis and Dunn's multiple comparison post hoc test results show significant differences from each respective data set through letter grouping system.

**Table 4.** Periodogram analysis for adult male *P. xylostella* locomotor rhythms. Chi-square periodogram analysis of 6-day data sets for the indicated number (n) of individual males under the indicated conditions. Relative rhythmic power (RRP) was calculated as the ratio between chi-square periodogram amplitude and significance threshold. # indicates that there was only a single rhythmic individual.

|  |  |  |  |
| --- | --- | --- | --- |
| **Male Condition (n)** | **% rhythmic** | **Period length (h)** | **RRP** |
| D/D  (23) | 4.3 | 32.5 | 0.85  ±.02a |
| 12/12  (33) | 84.8 | 23.9  ±0.1 | 1.31  ±.05bc |
| 6/6/6/6  (35) | 85.7 | 24.1  ±0.3 | 1.41  ±.09bc |
| 14/10  (20) | 75.0 | 23.9  ±0.1 | 1.22  ±.07b |
| 16/8  (27) | 85.2 | 24.0  ±0.1 | 1.41  ±.09bc |
| 18/6  (33) | 87.9 | 23.8  ±0.2 | 1.40  ±.07bc |
| 20/4  (28) | 96.4 | 24.0  ±0.1 | 1.72  ±.07c |
| L/L  (26) | 11.5 | 27.2  ±2.1 | 0.86  ±.03a |

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**Figure 3.** Phase-associated locomotor activity differences. Tufte slopegraph comparisons are shown for adult *P. xylostella* moth locomotor activity between (A) dark and light phases in 12:12 L/D cycles, (B) dark and subjective dark phases in DD cycles, (C) dark and light phases in 20:4 L/D cycles and (D) the 1st and 2nd 6:6 L/D phases in 6:6:6:6 L/D/L/D cycles. N indicates number of moths with the paired mean difference plotted on the right y-axis with bootstrap sampling distribution and 95% confidence interval indicated by the vertical error bar with two-sided permutation t-test *P* value.

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**Figure 4.** Average dark phase activity as a function of dark phase duration for P.xylostella adult male and female diel locomotor activity. The average ± SEM diel dark phase activity data is plotted for L/D cycles with 4, 6, 8,10 and 12h of dark as well as D/D cycles. The 12h data represents a combination of the 12:12 L/D and 6:6:6:6 L/D/L/D combinations. A linear regression fit is shown along with theoretical alternatives assuming near-complete light repression representing either a constant dark phase activity rate or a constant diel activity level.

To follow up on observations by Wang and colleagues [49], who reported weak but significant circadian rhythmicity for locomotor behaviour of adult male P. *xylostella* of the Geneva 88 strain under 20°C D/D conditions following prior L/D entrainment, we repeated D/D locomotor analysis for adult male *P. xylostella* of the ROTH strain at 20°C following L/D entrainment at the same temperature. Again, no behavioural circadian rhythmicity was detected (87.5% arrhythmicity; average RRP 0.82 ± 0.038; **supplemental Figure S3**).

3.3. Diel locomotor activity patterns of individual adult P. xylostella males under simulated field conditions.

To better assess diel *P. xylostella* locomotor behaviour under more natural combined light and temperature cycles, we recreated environmental cycles mimicking UK semi-field conditions recorded in April and June (see [63]). Diel locomotor activity patterns recorded for individual adult males exhibited apparent rhythmicity under both conditions with both light- and temperature-associated features. Peak activity occured soon following dark phase onset, whereas light phase onset triggered a reduction in activity (**Figure 5, Table 5)**. A significantly larger proportion of diel activity occurred in the dark phase under simulated April conditions, which is consistent with the longer dark phase under these conditions as observed dark phase activity rates were similar (**Figure 5**). A close-up of several graphs

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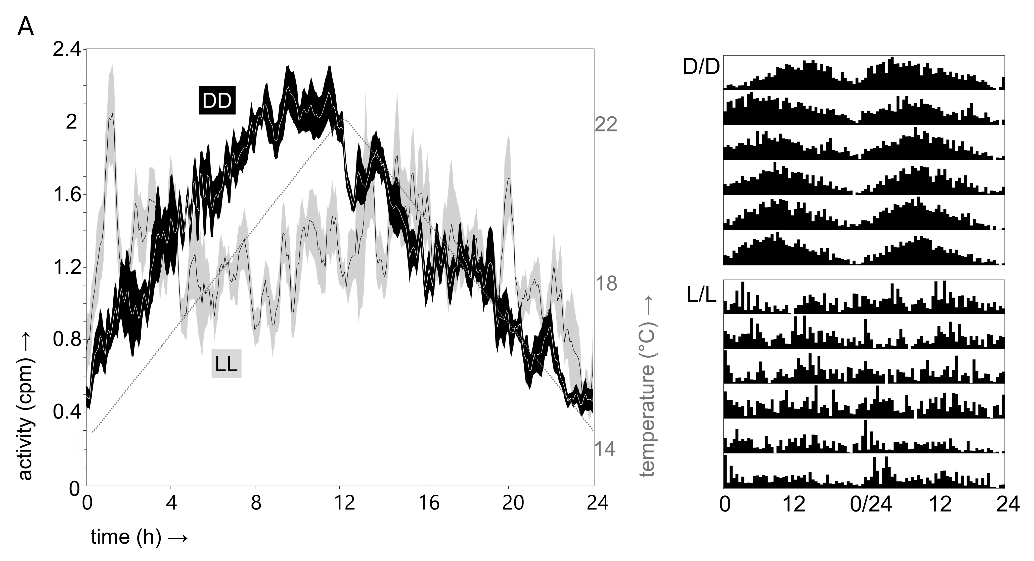
**Figure 5.** Male adult *P. xylostella* locomotor activity under UK April and June semi-field conditions. A. (Left) Average activity profiles show activity over 24 h of a 6 day average activity of 20 *P. xylostella* adult males. Time is plotted along x-axis in hours (h) starting at complete dark phase onset, with normalized amplitude along y-axis. Central line shows average activity with shaded area showing SEM. The black bar along the top of individual graphs shows dark phase with shaded bars showing light phase transition periods and a separate line to show temperature cycle with separate y-axis to the right. (Right) Double plotted actograms show average activity of moths over 6 days normalized against the next day’s activity in 30 minute bins of counts. The environmental light and temperature profiles recorded in the incubators are shown in Supplemental Figure S5.

**Table 5.** April and June semi-field conditions rhythmic data. % rhythmic equates to proportion of individuals with significant rhythmic period lengths calculated by periodogram with relative rhythmic power (RRP) then calculated by using the ratio between amplitude and significance threshold. Rhythm *P* equates to the *P* value of data’s fit to cosinudsoidal curve, Mesor equates to a rhythm-adjusted mean, Amplitude is a measure of amount of change between peak and trough of calculated curve, Phase peak equates to how many h after dark phase onset is the peak of fitted curve. \* indicates significant difference between April and June condition (n) repeat number, (h) hours.

|  |  |  |  |
| --- | --- | --- | --- |
| **Condition (n)** | **% Rhythmic** | **Period length (h)** | **RRP** |
| April  (20) | 90% | 24.1  ±0.1 | 1.68  ±.09 |
| June  (20) | 80% | 24.1  ±0.1 | 1.56  ±.13 |

3.4. Diel locomotor activity behaviour of individual P. xylostella males in response to temperature cycles in constant light or constant dark conditions.

Given the temperature-associated diel locomotor activity features observed under simulated field conditions, we considered the possibility that temperature cycles might induce diel locomotor rhythms in D/D and/or L/L conditions. Significant diel rhythmicity was observed in D/D conditions in the presence of a gradually ramping 14-22C temperature cycle, but this was not the case in L/L (see Figure 6, Table 7). Locomotor activity was strongly suppressed by the presence of constant white light and residual activity did not show diel rhythmicity for the large majority of adult male moths. In contrast, in D/D increased activity was associated with the 12h phase surrounding peak temperature as well as the 12h phase associated with rising temperatures.



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**Figure 6.** Adult male *P. xylostella* diel locomotor rhythms in D/D or L/L temperature cycle conditions.

**Table 6.** Constant dark and constant light temperature cycle data. % rhythmic equates to proportion of individuals with significant rhythmic period lengths calculated by periodogram with relative rhythmic.power (RRP) then calculated by using the ratio between amplitude and significance threshold. \* indicates significant difference between hot and cold condition (n) repeat number, (h) hours.

B.

|  |  |  |  |
| --- | --- | --- | --- |
| **Condition (n)** | **% rhythmic** | **Period length (h)** | **RRP** |
| 14-22 D/D  (32) | 75 | 24.08  ±.089 | 1.36  ±.090a |
| 14-22 L/L  (29) | 17 | 25.80  ±1.98 | 0.92  ±.021b |

4. Discussion

4.1. Clock gene regulation

Our findings that adult *P. xylostella* exhibit rhythmic diel and circadian cycling of *per* and *tim* matches observations for other Lepidoptera [64-67]. Noctural moths representing a variety of Lepidopteran families were previously shown to exhibit diel and circadian *per* and *tim* mRNA rhythms with peaks during (subjective) night. This includes the cotton boll-worm *Helicoverpa armigera* [67], mediterranean flour moth *Ephestia kuehniella*  [65], Chinese oak silk moth *Antheraea pernyi* [68] and silkworm *Bombyx mori* [69]. We can now add *P. xylostella* as a representative of the superfamily Yponomeutoidea to this list. Moreover, *per* and *tim* transcripts of the day-active monarch butterfly *Danaus plexippus* have also been found to exhibit similar rhythms in L/D and D/D [33,64] confirming that the circadian control of these transcripts is conserved and can be associated with Lepidoptera occupying different temporal niches [70,71]. Under D/D conditions both *per* and *tim* had lower amplitude in adult male *P. xylostella* relative expression values compared to L/D, with a significantly reduced rhythmic amplitude for *per* expression. Such damping of clock gene expression in D/D versus L/D conditions has been observed in other Lepidoptera such as *Ephestia kuehniella* [65] and may reflect both light-mediated enhancement of rhythms as well as loss of synchrony in peripheral clocks. Tissue-specific studies of molecular circadian rhythms under prolonged free-running conditions may clarify this.

4.2. Absence of circadian rhythms in P. xylostella adult locomotor behaviour.

Various aspects of Lepidopteran behaviour are known to exhibit control by the circadian clock (as reviewed by [72]). Notably, circadian regulation of adult locomotion or flight has been reported for *Ephestia kuehnellia, Antheraea pernyi, Hyalophoroa cecropia, Samia cynthia ricini*, *Manduca sexta* and *Hyles lineata* [65,73,74]. Though many studied Lepidoptera express rhythms in D/D, there are some that also show notable weakening of rhythms, including *Hyles lineata* [73] and female *P. interpunctella* [75]. We failed to observe circadian control of adult behaviour in *P. xylostella*. Under constant conditions adult moths failed to exhibit detectable circadian rhythmicity in their locomotor behaviour. This was true for both males and females at 17°C in D/D or L/L as well as males at 20°C in D/D. Moreover, there was no evidence of circadian modulation of diel behaviour, which can be evident by anticipatory increases in activity ahead of light/dark transitions [76] or circadian modulation of non-24h L/D cycles [62]. In fact, the complete absence of circadian rhythmicity also contrasts with a prior report of weak but significant circadian locomotor rhythms in adult male *P. xylostella* [49]. It should be noted a number of experimental differences may account for this discrepancy as our work differed in the strain used (ROTH versus Geneva 88), culture method (culture on *Brassica rapa* versus artificial diet) and there were also subtle differences in the adult setup for locomotor behaviour.

4.3. Light-mediated repression of P. xylostella moth locomotion.

Adaptations to avoid activity under light conditions represent a widely adopted survival strategy [77,78]. Avoiding these activities during light phases is common due to increased risks of predation under light exposure [79]. Moreover, key physiological processes in moths are known to be disrupted by artificial light [80-82]. Light avoidance is particularly well known among nocturnal moths [71] and it has been noted in the study of other behaviours in *P. xylostella* with over 70% of mating and oviposition taking place within the first half of dark phase in 16/8 conditions [83]. Consistent with these broader principles, our adult moth locomotor activity records demonstrated strong non-circadian responses to changes in lighting conditions with activity levels showing a sudden and sustained decrease upon exposure to light and an immediate temporary increase upon the onset of darkness. Our behavioural analyses under simulated field conditions with dimmable lighting are compatible with *P. xylostella* exhibiting a high level of light sensitivity with strong activity responses associated with subtle changes in light exposure at the beginning of dawn and the end of dusk. *P. xylostella* may preferentially avoid activity at dawn as predators may use morning light to hunt lingering nocturnal prey, with previous research showing higher predator foraging efficiency around this time [84].

4.4. Homeostatic regulation of P. xylostella locomotor activity.

Sleep is known to exhibit a homeostatic component that is independent of circadian control, where the possible accumulation of ‘sleep debt’ leads to organisms attempting to maintain a certain level of sleep on average [85,86]. Our analyses indicated homeostasis of diel locomotor activity levels in adult *P. xylostella* at 17°C across a range of photoperiods with only L/L conditions exhibiting a dramatic reduction in activity levels. This phenomenon may at least be in part accounted for by the tendency of adult *P. xylostella* to exhibit a temporary peak in locomotor activity shortly after the onset of darkness.

4.5. Temperature regulation of diel P. xylostella adult locomotor activity

Our results showed temperature-driven control of adult *P. xylostella* locomotor activity. This may be relevant to impact of both diel and seasonal environmental temperature rhythms. In addition, the impact of temperature on *P. xylostella* activity is relevant in relation to migration across temperature zones [87,88] and the impact of climate change [3]. Notably, the impact of temperature on locomotor activity was abrogated in L/L conditions, but appeared to persist in more complex environmental L/D cycles simulating field conditions. This observation could indicate that prolonged light exposure is required for this effect. In many animals prolonged exposure to L/L disrupts the circadian clock and associated behavioural rhythms. In insects such as the monarch butterfly *D. plexippus* [41] and fruit fly *D. melanogaster* [89] this molecular and behavioural arrhythmicity is mediated via the circadian photoreceptor dCRY. dCRY has an orthologue in *P. xylostella*, which was recently genetically disrupted [90]. It would be of interest to determine whether *dcry* mutant P. xylostella exhibite temperature-mediated modulation of locomotor activity in L/L.

4.6. Disassociation of locomotor behaviour rhythms and circadian clock function

It is unclear why *P. xylostella*’s locomotor activity is governed by light-mediated repression, temperature modulation and homeostatic regulation without exhibiting circadian rhythmicity. One possibility is that the ROTH strain used in this study lost some of its original circadian behaviour after decades of culture under laboratory conditions. Nevertheless, independent observations for the Geneva 88 strain also showed either arrhythmic or weakly circadian behaviour suggesting that *P. xylostella* locomotor behaviour may be largely controlled by alternative means [49]. Perhaps, a homeostatic mechanism in combination with responses to environmental cues is sufficient to drive the onset of temporary peaks in locomotor activity at the onset of darkness. Beyond abiotic time cues such as light and temperature *P. xylostella* may also respond to rhythmic signals emitted by its host plants. Studies have repeatedly indicated a close relationship between the circadian rhythms of Lepidoptera and host plants [91,92]. Therefore, further research on how *P. xylostella* interacts with its host plants is needed. In this context, it is of interest that *P. xylostella* sexual activity was altered in response to host plant volatiles [83].

It is possible that locomotor activity is an exception and that other relevant rhythmic adult behaviours including feeding, mating and oviposition are circadianly controlled [83,93]. Whether migration, which is photoperiodic in a number of insects [94], is controlled by the circadian clock in *P. xylostella* remains to be determined. As noted above, Campos and colleagues found that modulating photoperiod had no discernible effect on *P. xylostella* morphological and life history traits in a neotropical population [48]. It is possible that temperature rather than photoperiod acts as a migration cue due to its strong effect on the *P. xylostella* life cycle. In *D. plexippus*, a widely researched migratory lepidopteran, migration is triggered via cooling temperatures and is then maintained using circadian clock assisted compass mechanisms [41,95]. Insensitivity to photoperiodic entrainments may be advantageous to *P. xylostella*,opting for direct response to light conditions, due to the large changes in light conditions associated with the scale and speed of *P. xylostella* migration [8,96]. Along with temperature, the age and affected nutritional quality of host plants may act as a migration cue as it has been shown that older host plants produced *P. xylostella* adults with favourable migratory traits [97].

5. Conclusions

Circadian control of activity rhythms has been described at the behavioural and gene expression levels in both moths and butterflies however there can be noticeable species-specific differences between such [64,65,98]. It is therefore unclear how daily timekeeping acts in individual species. We confirmed light-entrained and circadian rhythms of the core clock transcripts *per* and *tim* that matched the behaviour of orthologous genes across Lepidoptera and other animals. This work could be further expanded by determining the circadian transcriptome of *P. xylostella* to further assess the relatedness of its clock mechanisms to those found across other animals. Our findings now provide evidence for uncoupling of molecular circadian rhythms from adult locomotor behaviour in the economically impactful pest *P. xylostella*. Instead, a combination of light-mediated repression, temperature-modulation and homeostatis were identified as important determinants of adult *P. xylostella* diel locomotor behaviour. Our use of simulated field conditions provides new hypotheses regarding the field activity of adult *P. xylostella* activity. Follow-up studies may link our results to temporal aspects of integrated pest management strategies for *P. xylostella*, such as those involving chemical treatments and trapping [83,99-102].

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